A Study of Diarrhoea among Children with Special Reference to Rotavirus including Detection and Molecular Characterization of Rotavirus in a Tertiary Care Hospital, Thanjavur

Dissertation Submitted to

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CERTIFICATE

This is to certify that the dissertation entitled **"A Study of Diarrhoea among Children with Special Reference to Rotavirus including Detection and Molecular Characterization of Rotavirus in a Tertiary Care Hospital, Thanjavur"** submitted to the Tamil Nadu Dr. M.G.R. Medical University, Chennai in partial fulfilment of regulations required for the award of M.D. Degree in Microbiology is a record of original research work done by **Dr. A. Shanthi** at the Department of Microbiology, Thanjavur Medical College and Hospital, Thanjavur during the period from July 2014 to June 2015 under my guidance and supervision and the conclusions reached in this study are her own.

Dean
Thanjavur Medical College

Signature of Guide
Professor and Head,
Department of Microbiology

DECLARATION

I, **Dr. A. Shanthi** truly declare that the dissertation entitled **"A Study of Diarrhoea among Children with Special Reference to Rotavirus including Detection and Molecular Characterization of Rotavirus in a Tertiary Care Hospital, Thanjavur"** submitted to the Tamil Nadu Dr. M.G.R. Medical University, Chennai in partial fulfilment of regulations required for the award of M.D. Degree in Microbiology is a record of original research work done by me at the Department of Microbiology, Thanjavur Medical College, Thanjavur during July 2014 to June 2015. I have not submitted this dissertation on any previous occasion to any University for the award of any degree.

Place: Thanjavur

Date: (Dr. A Shanthi)

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Thanjavur Medical College

THANJAVUR, TAMILNADU, INDIA-613 001

(Affiliated to the T.N.Dr.MGR Medical University, Chennai)

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Approval No. : 026

This is to certify that The Research Proposal / Project titled

A STUDY ON DIARRHOEA AMONG CHILDREN WITH SPECIAL REFERENCE TO ROTA VIRUS INCLUDING DETECTION AND MOLECULAR CHARACTERIZATION OF ROTA VIRUS IN TERTIARY CARE HOSPITAL, THANJAVUR.

submitted by Dr. A. SHANTHI ..... of

Dept. of MICROBIOLOGY .....Thanjavur Medical College, Thanjavur

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Thanjavur
Dated : 28.01.2014

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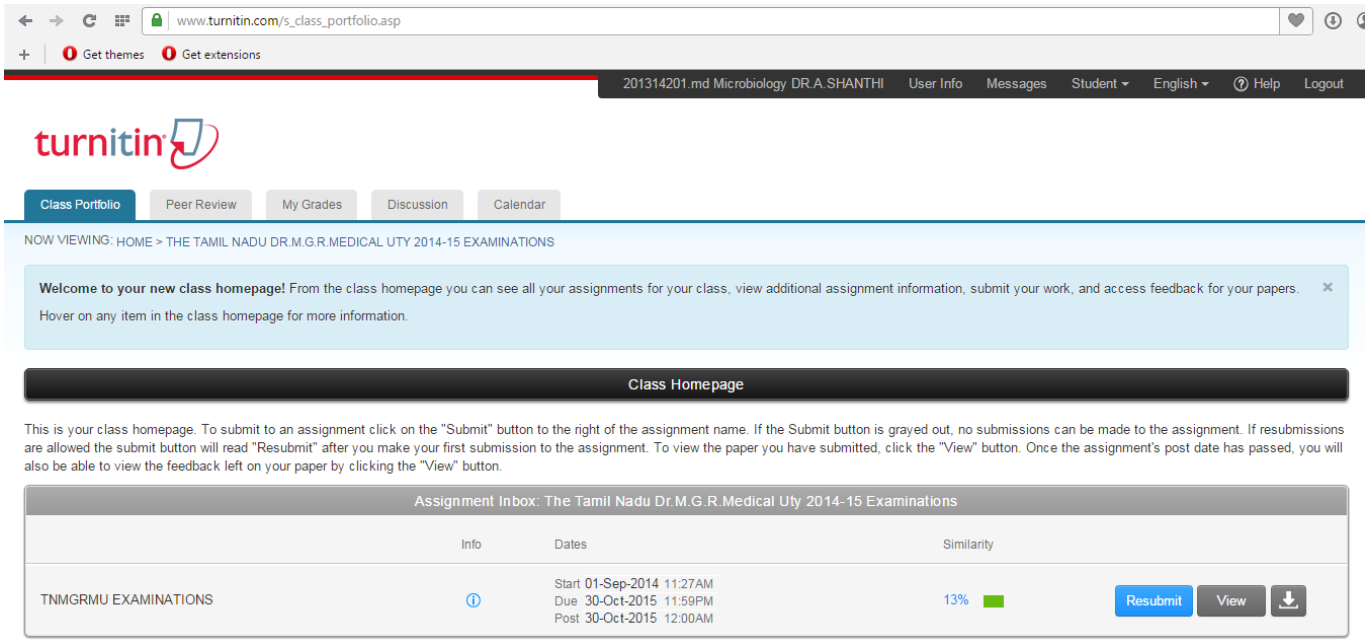

ABSTRACT

A STUDY OF DIARRHOEA AMONG CHILDREN WITH SPECIAL REFERENCE TO ROTAVIRUS INCLUDING DETECTION AND MOLECULAR CHARACTERIZATION OF ROTAVIRUS IN A TERTIARY CARE HOSPITAL, THANJAVUR

Aim and Objectives:

Diarrhoeal diseases are the second most common cause for mortality in children below five years. Currently Rotavirus is the leading common cause of severe, life threatening gastroenteritis in children. Rotavirus is the cause for 23-39% of diarrhoea in children. Prevalence of Rotavirus infections varies according to age, season and geographical area. The aim of this study to determine the Prevalence of Rotavirus infections, to characterize Rotavirus strains circulating during this period, provide region specific common genotype information of Rotavirus in Thanjavur tertiary care hospital.

Materials and Methods:

This study includes of 80 children aged less than 5 years suffering from acute gastroenteritis, admitted in pediatric ward, at tertiary care hospital Thanjavur Medical college Hospital, Thanjavur during July 2014 to June 2015. All the 80 samples initially tested to rule out bacterial

and parasite causes. By using Premier™ Rotaclone ELISA Kit Rotavirus antigen was detected. Randomly selected 15 samples including positive and negative samples were tested for RNA identification by real time multiplex RTPCR. Six positive samples by RTPCR were further processed for genotyping by conventional multiplex PCR.

Results and Observations:

Prevalence of Rotavirus diarrhoea in this study was about 29%. Rotavirus diarrhoea was more common in the age group of below 2 years. There was no significant difference between the sexes in this study. Rotavirus diarrhoea was common during winter months from October to March. 87% of RV positive children presented with vomiting which was considered as second predominant symptom next to diarrhoea. 58% of the cases reported with severe dehydration in this study. Duration of hospitalization in Rotavirus positive cases was more when compared to negative cases which imply severity of rotaviral infections. Out of 15 samples, 40% were positive by both ELISA & Rotavirus-A Real-time PCR, 46% were negative by both ELISA & Rotavirus-A Real-time PCR, 13% which were positive by ELISA were negative by RT PCR were considered as borderline positives.

G-P genotyping was done for 6 samples which were positive by Rotavirus-A Real-time PCR. Among 6 samples, G-P type combination most commonly found was G2P[4] (50%) followed by G1P[8] (33%). Remaining 17% was untypable.

Conclusion:

This study highlights the prevalence of Rota Viral Gastro Enteritis (RVGE) in under five year children which is 29%. ELISA for detection of rotaviral antigen is the very usual method for early diagnosis of RVGE. RT PCR is gold standard method for diagnosing RVGE but it's very expensive to undertake as routine diagnostic procedure. The commonest strain of Rotavirus pertaining to Thanjavur tertiary care hospital during the study period was G2P[8] followed by G1P[4]; hence pentavalent vaccine RotaTeq which contains G1 & G2 is preferred over monovalent vaccine. Implementation of effective control measures such as safe drinking water, proper sanitation and vaccination is very much needs to control the morbidity caused by RVGE in under five children.

Key Words: Rotavirus, Thanjavur, RTPCR, Genotyping

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DEDICATED TO

Shirdi Sai Baba

My inspirational brother Shankar

My Parents

My Family

&

Yuga

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ABBREVIATIONS

AAP	American Academy of Pediatrics
AGE	Acute Gastro Enteritis
ANOVA	Analysis of variance
bp	base pairs
cDNA	Complementary DNA
Ct	cycle threshold
DLPs	Double layered particles
DL-RIs	Double Layered Core Replication Intermediates
dNTPS	deoxynucleotide triphosphates
dsRNA	double stranded RNA
EDIM	Epizootic Diarrhoea of Infant Mice
EIAs	Enzyme Immunoassay
elF4G	Eukaryotic initiation factor 4G
ELISA	Enzyme Linked ImmunoSorbent Assay
EM	Electron Microscopy
FAM	6-carboxyfluorescein
HEX	Hexachlorofluorescein
ICMR	Indian Council of Medical Research
LA	Latex Agglutination
LR	Lactated Ringer's
NSP	Non Structural Proteins
NTC	no-template control
NTPase	Nucleoside-triphosphatase
NTPs	Nucleotide Triphosphates
ORS	Oral Rehydration Salts
ORT	Oral Rehydration Therapy
PAGE	Polyacrylamide gel electrophoresis
PCR	Polymerase Chain Reaction
PEG	Polyethylene Glycols

QS	Quantitative Standard
RER	Rough Endoplasmic Reticulum
RIs	Replication Intermediates
RNase	Ribonuclease
rRT PCR	real-time Reverse transcription polymerase chain reaction
RT-PCR	Reverse Transcription Polymerase Chain Reaction
RV	Rotavirus
RVGE	Rota Viral Gastro Enteritis
SAGE	Strategic Advisory Group of Experts
SERS	Surface Enhanced Raman Spectroscopy
SG	Sub Groups
SLPs	Single layered particles
SPSS	Statistical Package for the Social Sciences
ssRNA	Single Stranded RNA
TAE buffer	Tris base, acetic acid and EDTA buffer
Taq	Thermus aquaticus
TLPs	Triple Layered Particles
TMB	Tetra Methyl Benzidine
TSI	Triple Sugar Iron
UNICEF	United Nations Children's Fund
UV	Ultra Violet
VP	Viral Proteins
WHO	World Health Organization
Zn	Zinc

CHAPTER 1: INTRODUCTION

1.1 BACKGROUND

Diarrhoeal diseases are the second most common cause for mortality in children below five years.[1] Currently Rotavirus is the leading common cause of severe, life threatening gastroenteritis in children. Globally, Rota Viral Gastro Enteritis (RVGE) causes about 611,000 childhood deaths annually. More than 80% of these deaths occur in developing countries like India, Pakistan, Nigeria, Ethiopia and Congo Republic.[2] India alone contributes 22% (i.e.,) one-fourth of global deaths.[3]

In 2011, UNICEF states that under five mortality rate in India is 61 per 1,000 live births and under five Diarrhoeal mortality rate is 8 per 1,000 live births. The Million Death Study is a currently running project conducted by Registrar General of India and collaborators to determine causes of death in India for all ages.

According to this study, 23,152 deaths among children younger than 5 years were reported with 3,053 (13.2%) due to diarrhoea. During 2005, approximately 3,34,000 diarrhoeal deaths were reported nationally, which means 1 in 82 Indian children are dying from diarrhoea before the age of 5 years.

On the basis of Million Death Study data, Rotavirus was estimated to cause approximately 34% of all diarrhoeal deaths in this age group.[1 4] Rotavirus caused an estimated 1,13,000 deaths annually. It is estimated that one child in 242 cases died from Rotavirus infection before five years of age. Highest mortality rates were found in Indian states of Bihar, Uttar Pradesh and Madhya Pradesh, which in total amounts to >50% of deaths i.e., 64,400 nationally.[1]

Rotavirus is the cause for 23 - 39% of hospitalized diarrhoea in children. Rotavirus gastroenteritis is significantly more severe than non-Rotavirus gastroenteritis. And also

more than 60% of RVGE required hospitalization. More than 80% of RVGE cases were under two years of age.[5] Burden of Rotavirus in India implies importance of Rotavirus as an importance public health problem in under 5 years children. Indian Rotavirus strain surveillance network data recently emphasized the need for region specific genotyping information to study Rotavirus epidemiology and to monitor strain variation after vaccine introduction.[6] Nowadays intractable diarrhoeal diseases are treated with antibiotics, irrespective of causes. These misuses of antibiotics can be avoided if Rota viral diarrhoea is diagnosed early. Also knowledge of disease and seasonal prevalence of different pathogens in different regions is critical for management of outbreaks and planning and implementation of diarrhoeal control measures.[7]

The most effective method to prevent and to reduce Rotaviral disease burden is the Vaccination. World health organization's strategic advisory group of experts (SAGE) strongly recommends including Rotavirus vaccine in National Immunization Programme, where under 5 years mortality is more than 10%. Currently Rotavirus vaccine is not included in National Immunization Programme in India; therefore vaccine coverage is low which thereby leads to most children vulnerable to Rotavirus infection.[8 9]

The aim of this study is to determine the

(1) Prevalence of Rotavirus,

(2) To characterize Rotavirus strains circulating during this period,

(3) To provide useful epidemiological and clinical data for Rotavirus surveillance.

CHAPTER 2: REVIEW OF LITERATURE

2.1 HISTORY

For centuries, acute diarrhoea has been a major worldwide cause of death in young children. In 1963 *Adams* and colleagues by using Electron microscope they observed virus-like particles in intestinal tissue from mice infected with epizootic diarrhoea of infant mice (EDIM) virus.[10] After ten years of this invention, in 1973 *Ruth Bishop, Geoffrey Davidson, Ian Holmes,* and *Brian Ruck* identified abundant particles of a 'new' virus (Rotavirus) in the cytoplasm of mature epithelial cells lining duodenal villi, from the children admitted to the Royal Children's Hospital, Melbourne.[11] Biopsy of duodenal mucosa from nine of those children with acute non-bacterial gastroenteritis was examined under electron microscopy which revealed virus particles in epithelial cells. The viruses were suggested belong to the orbivirus group. This orbivirus is believed to have been an important cause of sporadic gastroenteritis in children in Melbourne during the 3 months of the survey during 1973.[12] In 1974 by the same scientists, they found particles resembling orbiviruses in electron microscopy of negatively stained faecal extracts in 11 out of 14 children with acute non-bacterial gastroenteritis, no such particles were observed in faecal extracts from 9 control children. They insist that the method of differential centrifugation for extraction of faeces is simple, quick, and more sensitive than duodenal biopsy for detecting the virus. They also states from their studies that excretion of virus reaches a peak level at the third or fourth day, and the virus has usually disappeared from the stools by the eighth day.[13] In 1974, *Flewett et al* discovered that there was association between viruses causing acute gastroenteritis in children and newborn calves, which were indistinguishable in size and shape. The convalescent sera from humans and calves agglutinated human and calf virus capsids in both directions in immunoelectron microscopy.[14] *Bishop et al* suggested that

those viruses should be named as Rotaviruses because of their short spike wheel like appearance. (Rota means wheel in Latin).[15]

In 1970s, by electron microscope proven studies of Rotavirus states that Rotavirus was the common cause for acute gastroenteritis in many countries like Australia, Britain, Canada, U.S.A., Singapore, Rhodesia, India, Norway, and Japan.[16] It is now known that infection with Rotavirus is nearly universal, with almost all children infected by 5 years of age.

A vaccine for Rotavirus gastroenteritis was first licensed in August 1998 called as RRV-TV, Rota shield which is a rhesus-based tetravalent Rotavirus vaccine but was withdrawn in 1999 because of its association with intussusceptions. Second-generation vaccines were licensed in 2006 called as RV5, RotaTeq which is a live vaccine contains five reassortant Rotaviruses developed from human and bovine parent Rotavirus strains and in 2008 another vaccine was licensed called RV1 Rotarix which contains one strain of live attenuated human Rotavirus type G1PA[8].[17]

2.2 ACUTE GASTROENTERITIS (AGE)

Acute gastroenteritis is the infection of gastro intestinal tract with sudden onset of diarrhoea and vomiting.[18]

Diarrhoea

Diarrhoea is defined as the passage of three or more loose or liquid stools per day or more frequent passage than in normal for that Individual. Diarrhoea is usually a symptom of an infection in the intestinal tract which can be caused by a variety of bacterial, viral and parasitic organisms.

Diarrhoea may be further defined by duration of illness, Acute if <2 weeks duration, persistent or chronic if >4weeks duration.[18]

2.2.1 Acute Diarrhoea

About 90% of Acute Diarrhoea is due to infectious causes, 10% is due to medications, toxic ingestions, ischemic and other causes.

Causes of Acute Diarrhoea:

In pediatric age groups:

Viral: Viral causes play a major role in pediatric Acute Diarrhoea. More than 20 different types of viruses cause gastroenteritis. Group A Rotaviruses are considered to be the main agents followed by Calicivirus, Adenovirus, Norovirus, Sapovirus and Astrovirus. Rotaviruses constitute about 35% of acute diarrhoea globally.

Bacterial: *Escherichia coli* are the next common pathogen in developing countries. Other causes are *Campylobacter, Salmonella, Shigella, Clostridium difficle* and *Vibrio cholera*.

Parasitic: *Cryptosporidium, Giardia Lamblia, Cyclospora Species, Entamoeba Histolytica,* etc.[19 20]

In travelers:

Most common due to Enterotoxigenic *E. coli*, *Campylobacter*, *Shigella*, *Aeromonas*, Coronovirus and *Salmonella*.[18]

In food poisoning:

Salmonella, *Campylobacter*, *Shigella*, Enterohaemorrhagic *E. coli*, *Bacillus cerus*, *Staphylococcus aureus*, Hepatitis A Virus etc.[18]

Immunodeficient persons:

Mycobacterium species, Cytomegalovirus, Adenovirus, Herpes simplex, *Cryptosporidium*, *Isosporabelli*, *Microsporida* etc.[18]

In Nosocomial infections:

Clostridium difficile is the most common cause for Nosocomial diarrhoea.[18]

2.2.2 Chronic Diarrhoea

Chronic Diarrhoea also known as persistent diarrhoea may be defined as diarrhoea which persists for >4weeks duration.

Causes of Chronic Diarrhoea:

Secretory causes:

Addisons disease, Tumours, Partial bowel obstruction, Idiopathic secretory diarrhoea, exogenous and endogenous laxatives etc.[18]

Osmotic causes:

Osmotic laxatives, Lactase deficiencies and Non absorbable carbohydrates.[18]

Inflammatory causes:

Crohn's disease, radiation injury, infections, food allergy, gastrointestinal malignancies, etc.[18]

Steatorrheal causes:

Intraluminal Maldigestion, Whipple disease, Celiac Sprue Abetalipoproteinemia, Ischemia etc.[18]

Iatrogenic causes:

Cholecystectomy, ileal resection, bariatric surgery, vagotomy etc.[18]

2.3 ROTAVIRUS

Rotaviruses belong to Genus Rotavirus and Family Reo viridae. They are non-enveloped double stranded Ribo Nucleic Acid viruses.[21] There are seven other genera in Reoviridae family. They are Phytoreovirus, Fijireovirus, Cyporeovirus, Aqua reovirus, Orbireovirus, Colti reovirus and Orthoreovirus.[22] Important features which classify Rotaviruses in separate genus are (a) mature virus particle has triple layered icosahedral protein layer, (b) from the smooth outer shell 60 protein spikes extends, (c) contains RNA dependent RNA polymerase, (d) viral genome contains 11 segments of double stranded RNA ds RNA,[23] (e) virus replication occurs in the cytoplasm of the infected cells, (f) exhibit unique feature in morphogenic pathway that is virus particles are formed by budding into endoplasmic reticulum,[10] (g) Rotaviruses of same group are capable of genetic ressortment, (h) virus cultivation in vitro is facilitated by proteolytic enzymes which enhances viral infectivity.[23]

2.3.1 Virion Structure

The morphology of virion structure is very much distinctive. Cryo Electron Microscopy and x-ray crystallography gives complete structural description which observes three types of particles. They are Triple layered particles, double layered particles and Single layered particles.[24 25 26]

The complete virus particle resembles that of wheel as it has short spikes and well defined smooth outer rim. The name Rotavirus was coined based on this factor (in Latin Rota means wheel).

Triple Layered Particles (TLPs) refers to the complete infectious particles. They are 70 - 100nm in diameter. In TLPs all six structural proteins is seen VP 1, 2, 3, 4, 6, 7. Biological properties of TLPs are infectious and it inactivates polymerase enzyme. **Double**

layered particles (DLPs) which lack outer shell, also known as rough particles because from their periphery trimeric subunits of inner capsid projects. In DLPs only four structural proteins is seen VP 1, 2, 3, 6. Biological properties of DLPs are non infectious and it's activate polymerase. **Single layered particles (SLPs)** or core are seen in aggregation and infrequently. In SLPs only three structural proteins is seen VP 1, 2, 3. Biological properties of SLPs are non infectious and inactivate polymerase enzyme.[23 27]

Figure 2-1: Virion Structure

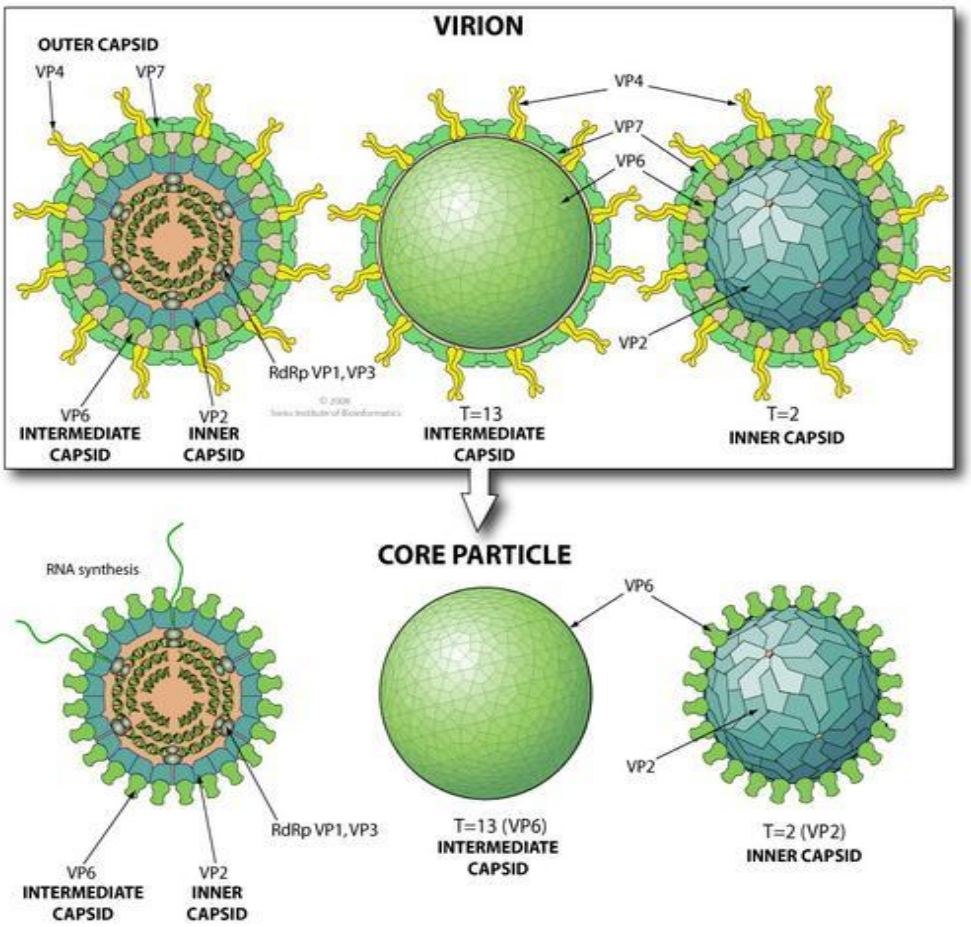
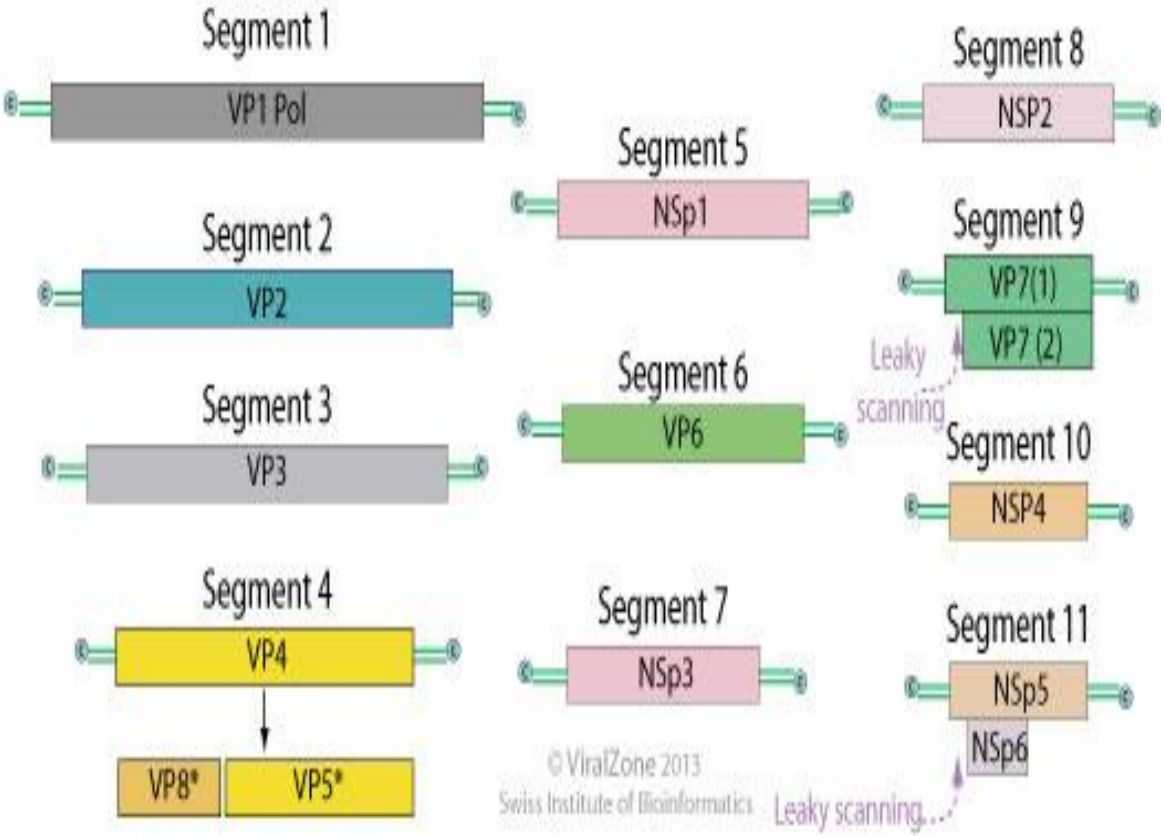


Figure 2-1 shows the virion structure of Rotavirus and it depicts three capsids outer capsid, intermediate capsid, inner capsid and also the core particle.

2.3.2 Genomic Structure

Genome of Rotavirus has 11 segments of Double stranded RNA (ds RNA) which is characteristic of this genus. Polyacrylamide gel electrophoresis easily separates these 11 segments which can be seen easily. The RNA segments codes for six structural proteins (VP 1, 2, 3, 4, 6, 7) and six non-structural proteins (NSP1-NSP6).[28]

Figure 2-2: Segments of Genome



Proteins of group A Rotaviruses:

Structural proteins:

- **VP1:**

  VP1 is coded by gene segment 1. VP1 is the largest polypeptide of Rotavirus which codes for the RNA polymerase.[23]

- **VP2:**

  VP2 is coded by gene segment 2. VP2 proteins form the Rotavirus core particle within which are enclosed the proteins VP1 and VP3 and the 11 genome segments. Next to VP6 strong immune response against VP2 common, IgG and IgA responses against them have nearly always been found in sera from individuals infected with Rotavirus.[23]

- **VP3:**

  VP3 is coded by gene segment 3. VP3, which is a guanylyltransferase, plays a role in RNA replication. VP3 showed affinity only for ssRNA, not for dsRNA.[23]

- **VP4:**

  VP4 is coded by gene segment 4. VP4 is a multifunctional spike protein with haemagglutinating, neutralizing and fusion activities and antigenic properties. VP4 has also been suggested to play a role in virulence. Based on VP4, P typing of Rotaviruses is done.[23]

- **VP6:**

  VP6 is coded by gene segment 6. VP6 forms the inner capsid of Rotaviruses. During Rotavirus infection the strongest immune response is usually formed to VP6.[23]

- **VP7:**

  VP7 is coded by gene segment 9. VP7 forms the outer protein layer of the Rotavirus. The protein was found to be glycosylated. VP7 glycoprotein was shown to be of high mannose type. Based on VP7 G typing of Rotaviruses is done.[23]

Non-structural proteins:

- **NSP1:**

  NSP1 is coded by gene segment 5. NSP1 recognize ssRNA in a nonspecific way, but with high affinity. NSP1 play a role in host range restriction along with VP7. It is interferon antagonist.[23]

- **NSP2:**

  NSP2 is a basic protein coded by gene segment 8. It localize in the viroplasms which are proposed sites of genome replication and suggested to play a role in RNA replication or packaging.[23]

- **NSP3:**

  NSP3 protein is coded by segment 7. NSP3 is also an RNA-binding protein. It binds 3' end of mRNA & cellular elf-4G. NSP3 inhibits host translation.[23]

- **NSP4:**

  NSP4 protein is coded by segment 10. Most studied nonstructural protein of Rotavirus. NSP4 was found to be glycosylated. Intracellular receptor for Double layered particles has role in morphogenesis of triple layered particles and modulates RNA replication. NSP4 was suggested to act as a viral enterotoxin that would trigger a signal transduction pathway.[23]

- **NSP5:**

  NSP5 is a nonstructural protein coded by segment 11. The protein is hydrophilic and rich in serine and threonine. Major component and forms viroplasm with NSP2 interacts with VP2.[23]

- **NSP6:**

  NSP6 is a nonstructural protein coded by segment 11. Self Association of NSP5 is regulated by NSP6 present in Viroplasms. Has function in RNA binding.[23]

Table 2-1: Genomic Structure of Rotavirus

Genome Segment	Protein Product	Location in Viral Particle	Number of Molecules / Virion	Function
1	VP1	Inner Core	12	RNA dependent RNA polymerase, ssRNA binding
2	VP2	Core	120	RNA binding, forms shell for the core
3	VP3	Inner Core	12	Guanylyl transferase, Methyl transferase, complex with VP1
4	VP4	Outer capsid (dimer)	120	P-type neutralization antigen, viral attachment, Hemagglutination, protease enhance infectivity
5	NSP1	Non structural		Binds RNA, Interferon antagonist
6	VP6	Inner capsid (trimer)	780	Group & subgroup specific, required for transcription
7	NSP3	Non structural		Binds 3'end of viral mRNA and cellular elF4G, Inhibits host translation
8	NSP2	Nonstructural		Binds RNA, forms viroplasms with VP1
9	VP7	Outer capsid (trimer)	780	Glycoprotein, G – type Neutralization &predominant antigen

10	NSP4	Nonstructural		Viral enterotoxin, intracellular receptor for DLPs, virulence
11	NSP5 NSP6	Nonstructural		Binds RNA, forms viroplasms

Source: Field's Virology, 6th edition.

2.3.3 Classification

Rotaviruses classified into Serogroups, sub groups, Serotypes and Genotypes based on serological schemes like immunological characteristics of different particles and genomic composition.

Serogroups:

There are seven serogroups (A to G). This serogrouping is based on antigenic epitopes on the internal structural protein VP6.[29] The groups are differentiated by lack of serological cross reactiveness of inner capsid protein VP6 with poly and monoclonal antibodies.[30] Cross reactive epitopes on VP6 are detected by ELISA. Group A, B, and C Rotaviruses found in both humans and animals. Groups D, E, F and G found only in animals.[23 28 30] Group B and C Rotavirus of animal origin have zoonotic potential.[31]

Group A Rotavirus is the cause for diarrhoea in infants and mammals. Group B Rotavirus is the cause for epidemics of severe adult diarrhoea. Group C is the cause for sporadic diarrhoea in children.[23]

Sub Groups (SG):

Subgroups are classified within group A Rotaviruses based on presence or absence of two distinct epitopes in VP6 and their reactions to one or both, or neither of two specific monoclonal antibodies (255/60, 631/9).[30] The subgroups are I, II, I +II, non I, and non II. Human Rotaviruses belong to SG II, while animal Rotaviruses belong to SG I.[32]

Serotypes:

Serotypes are the classification within each groups based on neutralization assays using hyper immune serum. This assay measures reaction of antibody against two outer capsid antigens VP7 & VP4. This assay differentiates VP7 as G types, as VP7 is a Glycoprotein and VP4 as P type, as VP4 is a Protease sensitive Protein. Currently there are 27 G serotypes and 35 P serotypes in group A Rotavirus.[23 28 29]

Genotypes:

Serogroups can also classify based on sequence analysis in to genotypes. VP7 genotypes and VP4 genotypes are determined by sequence analysis and hybridization analysis.[23] Reverse transcriptase PCR commonly used technique for VP4 & VP7 genotyping. Common combination of genotypes distribution worldwide was G1P[8], G1P[4], G2 P[4], and G4 P[8].[33]

G –type is indicated by single number (i.e.) G serotype and G genotype are numbered same. eg: G1, G2, G4 etc.

P-type is indicated by two numbers (i.e.) P serotype is indicated by a number and P genotype is indicated by a number in square brackets. eg: P1 [8], P1 [4] etc.[34]

2.3.4 Replication

Rotavirus replication occurs exclusively in cytoplasm of mature epithelial cell (i.e.) differentiated enterocytes at the tips of vili of the small intestine. In vitro replication was studied in immortalized monkey kidney cell lines (MA104, BS-C1) and secondary monkey kidney cells were there was similar replication as in humans.[28 35]

Steps in replication cycle:

Viral attachment:

Triple layered infectious Rotavirus particles attach to host cells surface through outer capsid protein VP4.[35]

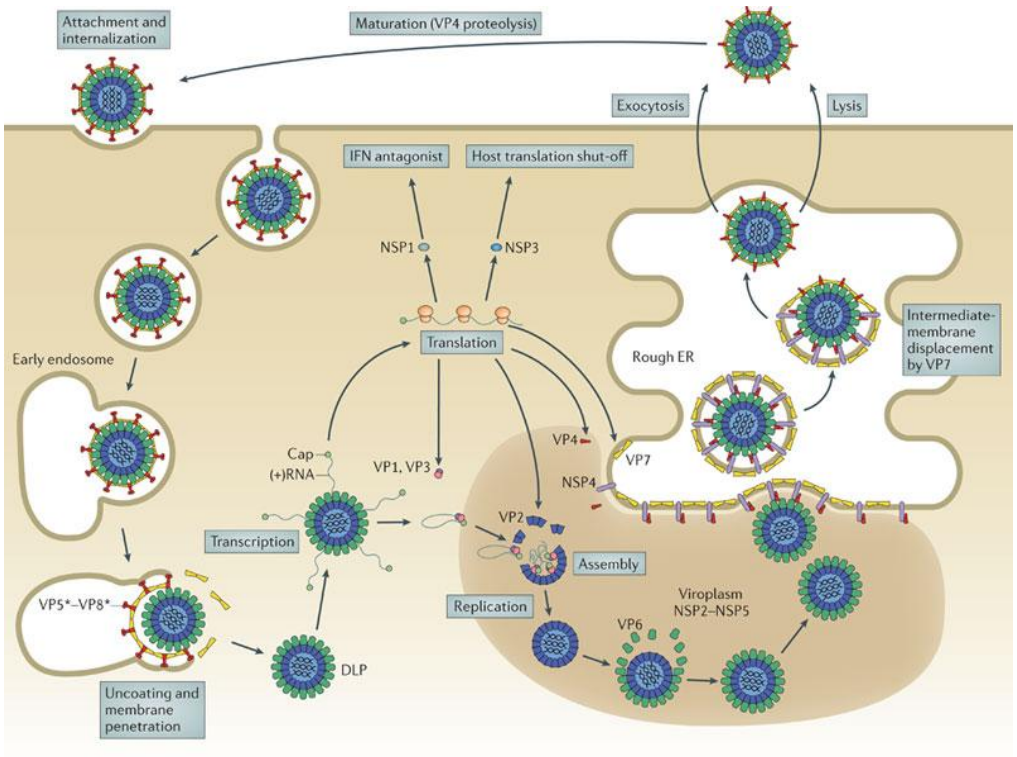
Viral Entry:

Virus entry into the cell by receptor mediated endocytosis or direct penetration. Lipophilic activity of body of VP4 spike is important factor for entry, also trypsinized particles enter the cells more rapidly.[36] Receptors for viral particles are sialic acid, glycolipids, galactose, and ganglioside receptor and heat shock cognate as co receptors.[23 37]

Uncoating:

After attachment and penetration, uncoating of outer capsid proteins occurs. Outer capsid proteins (VP4, VP7) are solublized in endocytic vesicle due to low concentration of calcium and it yields double layered particles.[23 38]

Figure 2-3: Replication of Rotavirus



Primary Transcription:

Transcription of minus strand double stranded RNA into mRNA takes place in cytoplasm. Primary enzyme involved in transcription is RNA dependent RNA polymerase (VP1). This enzyme acts as both transcriptase and replicase due to its specific structure. It has four distinct tunnels in its structure, (a) entry for templates[+]RNA or [-]RNA, (b) entry for NTPs, (c) exit for dsRNA/[-] RNA, and (d) exit for [+]RNA.

By VP1/VP2/VP3 complex multiple positive strands mRNA is transcribed from minus strand of dsRNA. These positive strand RNAs are exit from DLPs via aqueous channels.[23 39]

Translation:

Next step is the Synthesis of viral proteins from mRNA. NSP3 is involved in translation by binding to 3' end of mRNA and to eukaryotic translation factor elF-4G. These molecules pulled through VP2 by complex formation with NSP2 which has NTPase activity, NSP5 is also interact with this complex. Within the core, ssRNA replicated to form dsRNA genome. Interaction of VP6 with the core result in formation of Double layered particles consists of VP1, VP2, VP3, VP6 & non-structural proteins.[23 40]

Viroplasm:

Double layered particles accumulate and form pseudo crystalline aggregate termed as viroplasms. It is the site where incorporation of (+) RNA into replication intermediates (RIs) and virus assembly takes place. NSP2 and NSP5 played key role in viroplasm formation and function.[23 28 41]

Secondary transcription and translation:

From Double layered core replication intermediates (RIs) secondary transcription and translation takes place in cytoplasm. From these processes secondary double layered core replication intermediates (DL-RIs) are assembled in viroplasm.[23]

Budding and maturation:

NSP4 facilitates budding of DL-RIs from rough endoplasmic reticulum (RER). NSP4 act as intracellular receptors. In RER maturation completed when DL-RIs acquire VP7 & VP4 to form triple layered particles. During this process particles acquire transient membrane envelope, this envelope shed before complete maturation which unique characteristic of Rotavirus replication.[23 28]

Virus release:

Triple layered infectious virions are released by non classical vesicular transport mechanism bypasses Golgi apparatus and cell lysis.[42] VP4 interact with microtubule lipid network which destabilize epithelial cells facilitates Rotavirus exit.[43]

2.3.5 Pathogenesis of Rotavirus infection

Diarrhoea is major symptom of Rotavirus infection. Villi of the small intestine are infected by Rotaviruses. They multiply in the cytoplasm of enterocytes and damage their transport mechanisms. The mechanism of pathogenesis behind diarrhoea were (a) Reduction in total small intestinal surface area due to cell lysis, leading to a decrease in net fluid absorption (primary malabsorption) and lead to villous atrophy, (b) changes in the osmotic permeability of the mucosa secondary to cellular destruction, transepithelial resistance is decreased after Rotavirus infection result in increased epithelial permeability.[44] In young children Intestinal permeability has also been investigated using polyethylene glycols (PEGs). In acute phase of virus infection PEG absorption was

significantly lower than during 3 to 5 weeks after infection,[45] (c) Increased electrolyte secretion due to reactive crypt cell hyperplasia.[46] Diarrhoea caused by Rotaviruses may be due to impaired sodium and glucose absorption as damaged cells on villi are replaced by nonabsorbent immature crypt cells.

Another theory was enzyme lactase which is normally present in the lysozymes and in the brush border of intestinal villi. This enzyme in upper small intestine hydrolyzes the Lactose. When Rotavirus infects these cells which get damaged leads transient lactase deficiency. This pathogenesis cause increase in osmotic activity, increase in intestinal fluid, and causes bloating, distension, cramps, and finally watery diarrhoea. As the infected individuals had secondary lactose intolerance, sugar can be demonstrated in the stools.[47]

The major determinant of RNA segment responsible for pathogenesis is VP4. The other genes responsible for pathogenesis are VP3, VP7, NSP1, NSP2, and NSP4.[48] [49] NSP4 acts as enterotoxin considered as one of the important causes for pathogenesis of Rotavirus infection.[50] Signal transduction pathway is triggered which induces secretion. Into the lumen of the intestine damaged cells form slough and release large quantities of virus, which appear in the stool (up to 10^{12} particles per gram of faeces). Viral excretion usually lasts from 2 to 12 days. Intestine restores its normal function only after 3 to 8 weeks.[23]

2.3.6 Epidemiology

Epidemiological studies in developed and developing countries states that Rotaviruses were the major etiological cause for severe diarrhoea in young infants and children throughout the world.[23] Developed countries have a high morbidity rate but a low mortality rate, in contrast developing countries have high mortality rate due to poor vaccination. By age of 5, nearly every child worldwide will have had an episode of Rotavirus gastroenteritis. Epidemiology of Rotavirus serotypes is mostly done by

monoclonal antibody based ELISA for identification of VP7 type of clinical isolates followed by RT-PCR genotyping and nucleotide sequencing.[23]

G1, G2, G3, and G4 strains accounted for 97% of Rotavirus infections. From these G1 is more frequently detected strain in worldwide. Next to that G2 is more prevalent strain. Recently emerged strains during last decade were G9, G8, G5 & G12. G9 was first identified in Africa in 1997 and emergence of these new strains fears that current vaccine candidates may not protect against these new serotypes.[51]

Based on sequence analysis of VP4 type P1A[8] strains are most common strains in humans followed by P1B[4]. P[6] genotype has also been implicated in diarrhoea in children in Nigeria, Kenya, Brazil, Malawi, and India.[52] Four predominant combinations of P-G types were P[8]G1, P[4]G2, P[8]G3 and P[8]G4.[53]

In India, G1 strains were the most common except in western India, mostly mixed strains and non typable strains were common. In eastern India G9 strains were also noted. In northern India G2 strains are common when compared with other regions. In southern India G1 strains followed by G2 strains were common.[54] [55] Rare human G12 strains were also detected in Calcutta during routine surveillance in 2001.[56]

Transmission:

Rotaviruses are very contagious. They are generally transmitted by fecal-oral route. Also transmitted by respiratory route but it is not usual mode of spread. Duration of spread of virus from the stool is from 4–25 days of infection. Transmission is very efficient as these viruses are resistant to physical inactivation. The rapid spread of the infection is due to its ability to survive on various surfaces under different conditions. Recently there are speculations about role of animals as source of Rotavirus infection in humans. Human - bovine reassortant strains, human - porcine reassortant strains, human-simian reassortant strains have been endemic in India.[23] Rotavirus plays a role in causing Nosocomial

diarrhoea in children less than 5 years, it constitutes about 18% of Nosocomial diarrhoea.[57] Major risk factor in the incidence of AGE was considered to be Poor sanitation.[58]

Incubation period:

Incubation period is less than 48 hrs.[23 59]

Seasonal distribution:

Rotavirus infection is more common during winter months; this seasonal pattern is due to low relative humidity which facilitates the survival of the virus in surfaces.[23 60]

Age distribution:

Rotavirus infects primarily younger children and most commonly seen in age groups of 6 months to 2 years. Next highest frequency of illness is seen in age group of below 6 months. Rotavirus causes only 16% of gastro enteric illness in the age group of 5 years to 12 years.

In adults 4-7% of diarrhoea cases are due to Rotavirus. Group B Rotaviruses causes outbreaks in adults were documented in China and India. In adults mostly re-infection with Rotavirus is common. Asymptomatic and sub clinical manifestations are the clinical features in adults.[23]

Other disease associations include a possible role of Rotaviral infection induce two autoimmune diseases, type I diabetes and celiac disease due to molecular mimicry.[61]

2.3.7 Clinical features

RVGE produce wide spectrum of clinical features ranging from subclinical infection to mild diarrhoea to severe and fatal illness. Symptoms classically present with sudden onset, 1-2 days after infection. The clinical picture of RVGE is characterized by 4-7 days of acute febrile illness, vomiting, and watery, non-bloody diarrhoea.[62] If coexistence

of vomiting and diarrhoea means it leads to severe illness. Usually Rotavirus diarrhoea started later than vomiting but lasted longer.

Rotavirus (RV) antigenemia and RNAemia are common findings in Rotavirus-infected children (RV RNA and antigen in serum). It often correlates with more severe symptoms such as high fever, severe vomiting or convulsions.[63]

Blood urea nitrogen level is raised in >50% of cases. Urine specific gravity is raised in >70% of cases. Elevated liver enzymes in 20% of cases are noted.[23] Almost 60% of RVGE affected children are hospitalized which represent greatest medical burden of RV infection.[5] About 39% of diarrhoeal deaths in under five children in India are due to RV. Death occurred within 1-3 days of onset of symptoms. Factors which are responsible for fatal condition in various situations are severe dehydration, electrolyte imbalance and aspiration of vomitus. In developed countries, the mortality is low due to early medical intervention and aggressive rehydration therapy. RV rarely produces chronic infections,[23] but in Immunodeficient Children it may produce chronic infections.[64]

2.3.8 Immunity

Protective RV immunity is multi-factoral, obtain through secretary antibodies, humoral and cell mediated immunity. Natural immunity is acquired only after early and repeated exposure to the virus. Immune protections can either Homotypic which prevents infection with same virus or Heterotypic were prevention for different G types. Formation of IgA & IgG antibodies specific for VP7, VP4, VP6 and NSP4 is responsible for immunity. Strongest antibody response is against VP6.[62 65]

2.3.9 Laboratory Diagnosis

Direct detection of virus or viral antigen or virus specific serological response gives accurate diagnosis of RV infection.

Direct detection of virus:

Visualization of stool specimens for Rotavirus was done by EM – Electron Microscopy. It detects 80-90% of virus positive specimens when compared to other methods. Advantage is high specificity and rapid identification, as the Rotavirus has characteristic morphology. Disadvantage is that it requires expensive equipment. Also the method requires labor intensive and is relatively insensitive, requiring up to 10^6 intact viral particles per ml of stool. It cannot differentiate between sub groups of Rotavirus.[62 66]

Viral antigen detection methods:

Enzyme linked immunosorbent assay (ELISA) is the method of choice for diagnosis of RV. It detects VP6 group specific antigen in stool. ELISA detects as even as few as 10^5 to 10^6 viral particles/ml of stool. Antigen ELISA detection used as laboratory determinant for a case definition of RVGE and to find efficacy of RV vaccines. Monoclonal antibody-based enzyme immunoassay (Mabs) is more sensitive (95%) than Polyclonal antibody-based EIAs (75%).[67] ELISA for group A Rotavirus is commonly done, ELISA should be done in Non A Rotavirus cases to detect group B and group C Rotaviruses.[68] Quantification of Rotavirus in stool and serum can be done by ELISA on basis of standard-dilution plot of antigen in reference stool.[69] Advantage of ELISA test is that it is rapid, detects within four hours and has sensitivity of 97% and specificity of 96%.[70] ELISA would provide the most appropriate information to monitoring RV infection and consequently yield the clearest baseline information to assess immunization response.[71]

Co agglutination & Latex agglutination (LA) are other methods for detection of virus antigen. They are simple, rapid and inexpensive methods which are most widely used for mass screening and field studies in outbreaks. Co agglutination is more sensitive than Latex agglutination (LA).[72] As they have specificity of 100% they are nowadays used as screening procedure. But the sensitivity is only about 70% so they are not indicated in routine diagnostic purposes.[70 73]

Virus antibody detection:

Antibody detection is used to measure serologic response of Rotavirus infection. Antibody detection can be done in serum, saliva, duodenal fluids and stool. Rotavirus IgA in serum reflects the immunologic status of intestinal lumen. Currently complement fixatation test, IgA & IgG ELISAs and neutralization assays are used. These tests have more of epidemiological value than diagnostic value.[23]

Nucleic acid detection:

Polyacrylamide gel electrophoresis (PAGE) detects viral RNA segments as the stool specimen contains large quantities of viruses. By electrophoresis the negatively charged dsRNA 11 segments are separated according to their sizes. These particles are designated as Electropherotypes. These Electropherotypes patterns of dsRNA can be visualized in gel by silver nitrate staining. Electropherotypes provide information on genetic diversity of Rotavirus, heterogencity of circulating Rotaviruses and are useful in tracing spread through a population for epidemiological survey.[74] Specificity and sensitivity rates were 91% and 100% respectively.[75] Main advantage of PAGE is lack of ambiguity in the results and main disadvantage is detects only when there is 10^8 to 10^9 viral particles/ml of stool, time consuming and has lower sensitivity rate than ELISA.[70 76]

To overcome these factors Modified PAGE technique was discovered and found to be rapid, simple, reliable and less expensive technique.[77]

Nucleic acid amplification techniques:

Reverse transcriptase polymerase chain reaction (RT-PCR) is mainly applied for Typing of Rotavirus strains such as serotyping and genotyping. It is considered as gold standard and confirmative test for diagnosis of Rotavirus infections. Both conventional and semi quantitative real time PCR is available for detection of genes. For serotying real time RT PCR is used and for genotyping semi nested PCR is used. The sensitivity RT PCR is about 98.4% and specificity is about 100%. The main advantages of this method were it is useful in detecting extra intestinal viruses, to find correlation between disease severity and viral load, to verify intact RNA in extracts, able to detect any viral genomes of serogroups and have potential for identification of new reassortant or recombinant strains that are unable to be typed.[78] The main disadvantages were relatively expensive, labour intensive, not suitable as a routine diagnostic procedure.[79]

Newer technique:

Surface enhanced Raman spectroscopy (SERS) has been developed for rapid and sensitive detection of Rotavirus. The SERS method relies on the fabrication of silver nanorod array substrates for direct structural characterization of viruses.[80]

2.3.10 Treatment

The clinical assessment of dehydration is more important as a primary component in management of RVGE in children. Aim of treatment of RVGE is to replace fluids and electrolytes lost by vomiting and diarrhoea.

For clinical assessment of dehydration, currently two numerical scores are in use (i.e.) Clark and Vesikari Clinical Severity Scoring Systems.

Scoring Systems is based on seven parameters like maximum number of stools per day, Diarrhoea duration (days), Maximum number of vomiting episodes per day, Vomiting duration (days), Temperature, Dehydration and Treatment. Scoring such as 1, 2, & 3 were given based on above parameters. Severity is considered as Mild when the score is <7, Moderate when the score is 7-10, Severe when the score is ≥11.

Mainly these Scoring Systems is used to identify the primary endpoint in Rotavirus vaccine efficacy trials and severe RVGE.[81]

Diarrhoea is classified based on clinical features as diarrhoea with no dehydration, diarrhoea with some dehydration and diarrhoea with severe dehydration. Treatment modalities are decided based on above factors.

Oral Rehydration Therapy (ORT):

ORT with oral rehydration salts (ORS) solution is adopted by World Health Organization (WHO) and the United Nations Children's Fund (UNICEF) as the primary tool for fighting dehydration. Intervention with ORT the mortality rate of children under the age of five suffering from acute diarrhoea has fallen from 4.5 million to 1.8 million annually.[82] Various formulations of ORSs with added glucose or glucose substitute are effective in treatment of RV diarrhoea.

Low Osmolarity oral rehydration salts:

WHO recommends ORS with reduced sodium concentration (i.e.) Low Osmolarity Oral rehydration salts for non cholera diarrhoea. Recommended ORS contains 75 mEq/l of sodium, 75 mmol/l of glucose and total osmolarity 245 mOsm/l.[23] One of the variant of

ORSs is rice based ORS solution is more effective in decreasing total stool output, increasing absorption and retention of fluid an electrolytes. It is equally effective as glucose based ORSs.[23]

Zinc supplementation:

WHO recommended that zinc supplementation should be provided at a dose of 10-20 mg per day for 10-14 days.[83] Beneficial effects of Zn are it improves the therapeutic value of ORSs, significantly reduce the severity and duration of diarrhoea. Factors which makes zinc as beneficial are (a) improved absorption of water and electrolytes by the intestine (undefined mechanism), (b) faster regeneration of gut epithelium, (c) increased levels of enterocyte brush border enzymes and (d) enhances immune response leading to increased clearance of the pathogens responsible for and duration diarrhoea from the intestine.23

Intravenous therapy:

Is recommended for severe dehydration, shock for those not responding to ORSs and for those who have contraindications for ORSs such as depressed consciousness. Recommended solutions are Lactated Ringer's (LR) solution, normal saline, or a similar solution should be administered as 20 mL/kg body weight as an initial phase of management follower by maintenance phase.[84]

Use of loperamide, anticholinergic agents, bismuth, adsorbents and probiotics for management of acute diarrhoea in less than 5 years is not recommended by the American academy of pediatrics (AAP). Chronic RVGE in immunodeficient children are successfully treated by using human milk with RV antibodies.[23]

Newer modalities of RVGE management which are under trials were (a) bovine colostrums with RV antibodies, (b) oral gamma globulins, (c) oral human serum

immunoglobulin, (c) bovine colostrums from immunized cows with human RV strains, (d) antiviral agents, (e) adenosine analogs as anti Rotavirus activity, (f) raceadotril, an enkephalinase inhibitor, (g) nitazoxanide as anti infective drug, (h) use of probiotics.[23]

2.3.11 Prevention

Since traditional measures like sanitation, safe water supply and good hygiene practice have little influence on RV infection prevention, breast feeding provides protection only for a limited period during infancy and oral rehydration therapy is difficult in view of associated vomiting episodes; vaccine is the choice for effective Rotavirus prevention.[2]

Rotavirus vaccines are efficacious against both Rotavirus morbidity and mortality and have the potential to substantially reduce child mortality in low-income countries if implemented appropriately.

Rotavirus Vaccines:

Currently, two live attenuated oral vaccines are available **Rotarix (RV1)** and **RotaTeq (RV5).**

Rotarix (RV1):

Contains one strain of live attenuated human Rotavirus Type G1PA[8]. It is orally administered in two doses at 6-12 weeks of age, with an interval of 4 weeks between 1st and 2nd doses.[2 17]

RotaTeq (RV5):

Contains five reassortant (i.e.) pentavalent vaccine, developed from human and bovine parent Rotavirus strains containing G1-G4 and P1A [8] antigens. It is orally administered in three doses starting at 6–12 weeks of age, with the subsequent doses

administration at 4–10 weeks interval, and the third dose administered before 32 weeks of age.[2 17]

Recently many studies in countries like United States, Finland were done to find the efficacy of RV vaccines in children which states that there was significant reduction in outpatient AGE visits and hospital admissions due to RV in the 2-year post immunization period. Rotavirus vaccine reduces very severe Rotavirus infections by 74% and reduces Rotavirus hospitalizations by 57%.[85] WHO recommends that even a RV vaccine with approximately 60% efficacy may be given as it has the potential to save more than 1.5 million lives in Africa and Asia alone, between 2010 and 2025.[2]

Rotavirus vaccine is currently intensively targeted for inclusion in national immunization programme in India, in 2009 WHO recommends including Rotavirus vaccines in the national immunization programs in developing countries.[86] Indian government recently launched ROTAVAC vaccine developed completely in India. It is a monovalent human-bovine Rotavirus vaccine and developed from new strain 116E which completed phase III trial. Result of the trials states that it very effective and well tolerated in Indian infants.[87]

2.3.12 Indian Rotavirus Strain Surveillance Network

In December 2005, the Indian Rotavirus Strain Surveillance Network was established by Indian Council of Medical Research (ICMR). Goal is to generate geographical representative information on the clinical, epidemiological, and virological features of severe Rotavirus disease in Indian children, with use of standardized protocols for enrollment and diagnostic evaluation. The Indian Rotavirus Strain Surveillance Network now established with 8 regional laboratories, four referral laboratories and 30 hospitals in 8 different regions of India.[88] Two years of surveillance report highlights that

there is high prevalence of Rotavirus gastroenteritis in India, the higher severity of Rotavirus disease than that of other diarrhoeal diseases, and the circulation of a diverse range of Rotavirus strains, including several uncommon and emerging strains. These data highlight the need for development and implementation of effective prophylactic measures, such as vaccines as National Immunization Programme, to prevent the large burden of Rotavirus disease among Indian children.[89]

CHAPTER 3: AIM OF THE STUDY

3.1 AIM

To examine the prevalence of Rotaviral infection in children below five years of age in tertiary care hospital, Thanjavur.

3.2 OBJECTIVES

1. To find out commonest cause of diarrhoea in children in tertiary care hospital, Thanjavur.
2. To detect Rotaviral antigen by ELISA method in stools samples of all children under study.
3. To examine prevalence of Rotaviral infection in children with diarrhoea and provides comparison of prevalence of Rotavirus infection with that of non Rotaviral infection.
4. To monitor the disease burden associated with Rotavirus.
5. To confirm the Rotaviral detection with molecular detection method by real time Polymerase Chain Reaction.
6. To characterize and determine genotypes of the Rotavirus strains in those children and provide region specific common genotype information in Thanjavur tertiary care hospital.

CHAPTER 4: MATERIALS AND METHODS

4.1 STUDY DESCRIPTION

Place of study	Thanjavur Medical College Hospital, Thanjavur
Study period	One year (July 2014 to June 2014)
Collaborating Department	Department of Pediatrics
Design of Study	Prospective and Observational study
Ethical Committee	Prior approval obtained from Ethical Committee Clearance
Informed Consent	Obtained from each patient's caregiver / parent
Sample	Stool specimen

A total of 80 children aged less than 5 years suffering from acute gastroenteritis, admitted in pediatric ward, at tertiary care hospital Thanjavur Medical college Hospital, Thanjavur, were included in the study during period from July 2014 to June 2015. Institutional ethics committee approval for the study was obtained before initiation of the study. The children with AGE, who met the inclusion criteria, and whose caregivers gave informed consent, were enrolled. (Refer APPENDIX 2)

Single faecal specimen was collected from each patient within first to fourth day of illness. So, totally 80 samples were collected for the study.

History of diarrhoea, its duration, its nature and associated symptoms such as vomiting, fever and other clinical symptoms were obtained from each child's parents or caregiver. Furthermore, basic demographic characteristics such as age, sex, place of domicile, socio-economic status were also obtained. Physical examination of children was conducted to determine associate clinical features such as pyrexia, low volume pulse, tachycardia and state of hydration. (Refer APPENDIX 3)

The samples were processed in the Central Service Laboratory, Microbiology Department and Thanjavur Medical College Hospital.

For collection of the sample the following inclusion and exclusion criteria were considered.

4.1.1 Inclusion Criteria

- Children below five years admitted for acute gastroenteritis.

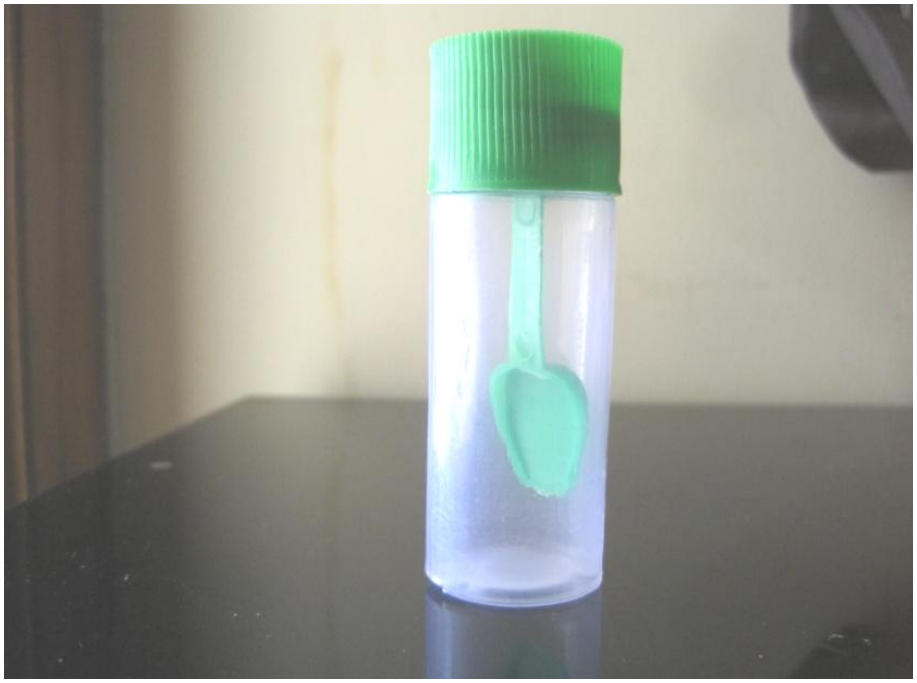
4.1.2 Exclusion Criteria

- Stools of children showing the presence of blood and mucus (dysentery).
- Diarrhoea due to food poisoning in children.
- Chronic or prolonged diarrhoea in children.
- Adolescents and adults are excluded from this study.
- Rectal swabs.

4.2 SPECIMEN COLLECTION

About 10 -15 ml of freshly passed stool samples is collected in sterile, wide mouth, leak proof plastic container from each child under study. Stool samples were collected within 7 days of onset of symptoms. After the collection, the stool samples were immediately transported to the laboratory for further processing. Collected specimens are stored in deep freezer at -20^{O}C until tested for Rotavirus antigen.

Figure 4-1: Sterile Stool Sample Container



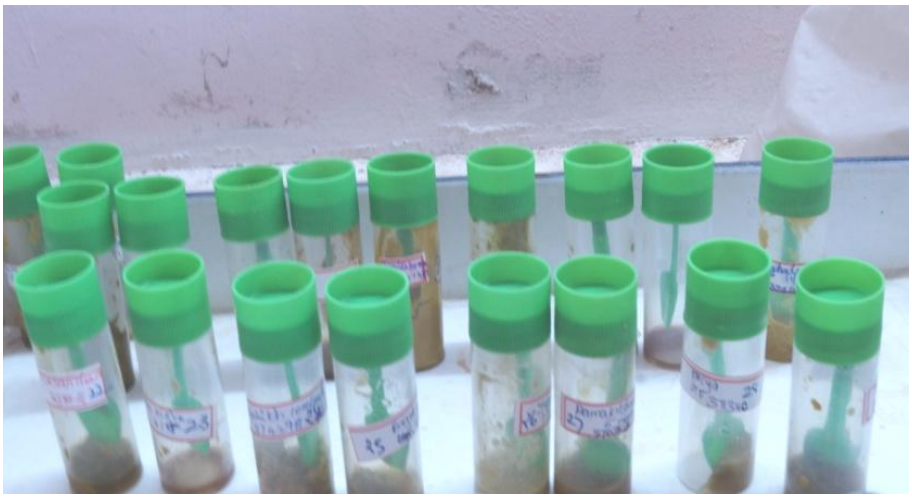


Figure 4-1 shows that that sterile, wide mouth, leak proof plastic container with scoop that was provided to collect stool samples for processing. Picture below shows some of the samples with labels.

4.3 SPECIMEN PROCESSING

The stool samples were immediately transported to the laboratory for further processing. Initially faecal samples are subjected to Macroscopic examination and microscopic examination for Colour, Consistency and Atypical components like mucus, blood and parasites. Then the samples are subjected to microscopic examination by saline and iodine wet mount for RBCs, Leucocytes, and Parasitic ova & cysts.

4.4 MACROSCOPIC EXAMINATION OF STOOL

A macroscopic examination of the received stool samples was conducted as per following criteria.

- Colour
- Consistency (formed / semi formed / liquid)
- Presence of blood
- Presence of mucus
- Presence of parasites.

4.5 MICROSCOPIC EXAMINATION BY SALINE AND IODINE WET MOUNT

4.5.1 Saline wet mount

Saline wet mount is prepared by mixing the stool with physiological saline. It is colorless preparation which highlights the staining property of egg**,** detects motility of trophozoites and other structures like pus cells, RBCs.

Procedure:

In clean glass slide add a drop of saline ,take match stick head size stool specimen and emulsify it in the drop of saline, put a cover slip over the saline suspension of stool. Examine the slide first under 10 x and then 40x objective of the microscope.

4.5.2 Iodine wet mount

Iodine wet mount is prepared by mixing the stool with iodine (Refer APPENDIX 1). It is brown colored preparation which highlights the presence of nuclei and glycogen mass in cyst.

Procedure:

In clean glass slide add a drop of Lugol's iodine, take match stick head size stool specimen and emulsify it in the drop of Lugol's iodine, put a cover slip over the iodine suspension of stool. Examine the slide first under 10x and then 40x objective of the microscope.

4.6 BACTERIAL CULTURE

The faecal specimens were subjected to culture in order to look for common enteropathogens. The stool sample was inoculated in MacConkey plates and incubated for 18 to 24 hours at 37˚C. At the end of the incubation period, the plates were examined for the isolates.

Bacterial isolates were identified by adopting the procedures of Gram staining, motility and routine biochemical reactions like Catalase test, oxidase test, motility test was done followed by Indole test, Methyl Red Test, Voges Proskauer Test, Citrate test, Urease test, nitrate reduction test, TSI test were done and results were recorded. (Refer APPENDIX 1) Based on these tests, the organisms were identified. Quality control was also performed for all the standard tests.

4.7 ROTAVIRUS DETECTION

Rotavirus antigen detection was done by ELISA method, Rotavirus molecular level RNA detection was done by Rotavirus-A Real-time PCR assay and Rotavirus genotyping was done by conventional PCR.

4.7.1 Rotavirus antigen detection by ELISA

Totally 80 samples were tested in batches.

Principle:

Detection of Rotavirus antigen was investigated by Enzyme immunoassay (EIA) using ELISA kit Premier^{TM} Rotaclone manufactured by Meridian Bioscience, Inc. Cincinnati, Ohio, United States. EIA utilizes monoclonal antibodies in a solid phase sandwich type ELISA. Plastic microtitre wells are coated with monoclonal antibody directed against VP6 which is group specific antigen of human Rotaviruses. Faecal suspensions, positive control and negative control were added to the wells and incubated simultaneously with an anti-Rotavirus monoclonal antibody conjugated to horseradish peroxidase, resulting in the Rotavirus antigen being sandwiched between the solid phase and enzyme linked antibodies. After 60 minutes incubation at room temperature, the sample well is washed in order to remove unbound enzyme labeled antibodies. Enzyme substrate A (urea peroxide) and substrate B Tetra Methyl Benzidine (TMB) are added to the wells and incubated for 10 minutes at room temperature. The enzyme bound in the wells converts the colourless substrate to blue colour. The intensity of the blue colour is directly proportional to the concentration of Rotavirus antigen in the sample. Addition of stop solution converted the colour from blue to yellow. Wells were read spectrophotometrically using a 450nm filter.

Figure 4-2: ELISA Kit for Rotavirus Antigen Detection

Figure 4-2 shows the ELISA kit Premier^{TM}Rotaclone manufactured by Meridian Bioscience, Inc. Cincinnati, Ohio, United States.

Reagents and Materials:

Following reagents and materials were used for the test:

- Rotavirus monoclonal antibody coated microtiter wells.
- Rotaclone conjugate contains horse radish peroxidase conjugated to anti Rotavirus monoclonal antibody in buffered protein solution with gentamycin and 0.02% thimerosal as preservatives.
- Positive control was inactivated simian Rotavirus SA-11 in buffered saline with 0.02% thimerosal as preservatives.
- Negative control was sample diluents.
- Sample diluent contains buffered saline 0.02% thimerosal as preservatives.
- Part A substrate buffer contains urea peroxide.
- Part B substrate solution contains Tetramethylbenzidine (TMB).
- Stop solution contains $1NH_2SO_4$.
- Sample transfer pipettes.
- Microtiter well holder.
- Distilled water
- Test tubes & test tube racks
- Absorbent paper
- Micropipette tips for 100µl & 1000µl
- ELISA microwell plate reader capable of reading absorbance at 450nm.

Reagent preparation:

- All reagents brought to room temperature before the procedure as per manufactures guidelines.
- All the reagents returned to 2-8 degree after use.

Specimen preparation:

- 1 ml of sample diluent was taken in test tubes for each stool sample.
- Stool samples were taken by transfer pipettes provided in the kit. Samples taken in quantity indicated by a mark in the transfer pipettes.
- Sample was resuspended in 1ml of sample diluent.

Procedure:

- Microtitre wells were taken, in appropriate wells marked for positive control, negative control & sample numbers.
- 100µl of diluted samples, positive control and negative control were added in the appropriate wells.
- 100µl of enzyme conjugate were added to each well. Mixed it gently.
- Incubated at room temperature for 1 hour.
- All the wells washed with distilled water thoroughly for five times.
- 100µl of substrate A solution were added to each well.
- 100µl of substrate B solution were added to each well.
- Incubated at room temperature for 10 minutes.
- After 10 minutes visual determination of all the wells were noted for colour change.
- 100µl of stop solution were added to each well for spectrophotometric determination.
- Absorbances of each well were read at 450nm by ELISA reader.

Interpretation of results:

Positive results by visual determination: Done before adding stop solution. Samples with blue colour are more intense than that of the negative is considered positive. Sample with colour equal to or less intense than the negative control is considered negative.

Positive results by spectrophotometric determination: Specimens with absorbance units (A_{450}) greater than 0.150 are considered positive .specimens with absorbance equal to or less than 0.150 are considered negative.

4.7.2 Molecular Detection Method: Rotavirus-A Real-time PCR assay

Randomly selected 15 samples, including 8 samples which are positive for Rota antigen and 7 samples which are negative for Rota antigen were tested for Rotavirus-A Real-time PCR assay.

a) Materials required:

Rotavirus-A Real-time PCR was done by using:

- Total RNA purification kit (PureFast® Total RNA Minispin purification kit)
- First cDNA synthesis kit
- Rotavirus-A Real-time PCR kit from HELINI Biomolecules, Chennai, India

b) Procedure for Viral RNA Extraction by spin column method:

- Done by using PureFast® Total RNA Minispin purification kit
- Transferred 100mg of Stool samples into 2ml centrifuge tube
- 1.6ml of Stool Buffer added and vortex thoroughly
- Incubated at room temperature for 5 min
- Centrifuge at 13000rpm for 5 min
- Transferred supernatant into fresh 1.5ml tube using micro pipetting
- 0.6ml of supernatant is transferred into another 1.5 millilitre centrifuge tube
- Added 600µl of Viral lysis buffer, 5µl of internal control template and 20µl of Proteinase K, Mixed well by inverting several times
- Incubate at 56ºC for 15min

- Added 600µl of Ethanol and mixed well
- Transferred 600µl of sample into the spin column. Centrifuged for 1 min. Discard the flow-through and place the column back into the same collection tube
- Repeated for remaining supernatant
- Added 500µl Wash buffer-1 to the spin column. Centrifuge for 30-60 seconds and discard the flow-through. Place the column back into the same collection tube
- Added 500µl Wash buffer-2 to the spin column. Centrifuge for 30-60 seconds and discard the flow-through. Place the column back into the same collection tube
- Discard the flow-through and centrifuge for an additional 1 min. This step is essential to avoid residual ethanol
- Transferred the spin column into a fresh 1.5 ml micro-centrifuge tube.
- Added 60µl of Elution Buffer to the center of spin column membrane.
- Incubate for 1 min at room temperature and centrifuge for 2 min.
- Discard the column and store the purified viral nucleic acid at -20°C.

c) First strand cDNA Synthesis:

The following reagents were added into a sterile, RNase-free tube on ice in the following order:

- 6µl of HELINI 5X cDNA Reaction Mix was added first which contains Reverse Transcriptase buffer, dNTPS and additives
- 2µl of Reverse transcriptase & RNase Inhibitor enzyme Mix was added next
- 6µl of Template RNA was added next

- 4µl Gene specific Primer Mix 2p/ul was added next,
- Total volume of above mix was about 20µl.
- Mixed gently and centrifuge.
- Incubated in thermal cycler for 60min at 42ºC.

d) Rotavirus-A Real-time PCR assay detection Protocol:

- 10µl of Probe PCR Master Mix was added to PCR vial. Probe PCR Master Mix contains 1.5U of Hotstart Taq DNA polymerase, 10X Taq reaction buffer, 3.5mM MgCl2, 1µl of 10mM dNTPs mix and PCR additives.
- 2.5µl of Rotavirus-A Primer Probe Mix was added next.
- 2.5µl of Internal control Primer Probe Mix was added next.
- 2.5µl of First strand cDNA was added next.
- 7.5 µl of nuclease free water was finally added to the PCR vial.
- Total volume of above mix was about 25µl.
- Centrifuge PCR vials briefly before placing into thermal cycler.
- Preparation of Negative Control by adding 10µl of nuclease free water instead of Purified RNA sample.
- Preparation of Positive Control by adding 10µl of four Quantitative Positive controls i.e.

  QS1-500000 copies

  QS2-50000 copies

  QS3-5000 copies

  QS4-500 copies
- PCR vials were placed into thermal cycler and programmed it as follows;
  - First step was Taq enzyme activation for 15min at temperature of 95ºC.

- Second step was Denaturation for 20sec at temperature of 95ºC.
- Third step was Annealing for 20sec at temperature of 56ºC.
- Data collection was done during this step.
- For data collection FAM channel was used for Rotavirus. FAM was 6-carboxyfluorescein labeled for first probe. And HEX Channel which is a reporter dye was used for internal control.
- Final step was Extension for 20sec at temperature of 72ºC.
- Denaturation, Annealing & Extension were set for 45 cycles.

4.7.3 Rotavirus-A virus Genotyping

All the six samples which were positive in rRT PCR were tested for genotyping by conventional PCR method.

a) Materials required:

- Purefast Viral DNA purification kit.
- PCR Master Mix.
- Rotavirus-A Genotyping PCR kit are from HELINI Biomolecules.
- Agarose gel electrophoresis consumables.

b) Methods :

G and P typing was carried out by amplification of variable sequences of the VP7 and the VP4 genes with eight G- and six –P type-specific primers (multiplex PCR) For Rotavirus-A Genotypes - G (VP7), two sets of specific primers were taken set1 and set 2. In set 1, following specific primers with recommended basepairs were taken.

Set-1:

G1 - 215bp

G2 - 351bp

G3 - 451bp

G4 - 582bp

G8 - 676bp

In set 2, following specific primers with recommended basepairs were taken.

Set-2:

G9 - 387bp

G10 - 250bp

G12 - 550bp

For Rotavirus-A Genotypes - P (VP4), following one set of specific primers with recommended basepairs was taken.

P4 - 369bp

P6 - 580bp

P8 - 250bp

P9 - 287bp

P10 - 398bp

P11 - 563bp

C) Genotyping cDNA Protocol:

- **Components required:**
  - cDNA reaction Mix
  - RT-Enzyme mix
  - Genotype Primer Mix
  - Purified Viral RNA
- **Procedure**
  - Three PCR tubes for each sample [for six Samples] were taken.
  - Tube 1 was for Genotype G set-I

- Tube 2 was for Genotype G set-II
- Tube 3 was for Genotype P
- 6 µl of cDNA reaction Mix was added in all 3 tubes
- 2 µl of RT-Enzyme mix was added in all 3 tubes
- 4 µl of Genotype Primer Mix was added in all 3 tubes
- 8 µl of Purified Viral RNA was added in all 3 tubes
- Finally Total reaction volume in all 3 tubes was about 20µl
- PCR vials was Centrifuged briefly
- These PCR vials are placed into thermal cycler
- Incubated at 42°C for 1hour

d) PCR Protocol:

- **Components required:**
  - **RedDye PCR Master -** It contains 2U of Taq DNA polymerase, 10X Taq reaction buffer, 2mM MgCl2, 1µl of 10mM dNTPs mix and PCR additives.
  - Genotype Primer Mix
  - Water
  - cDNA
- **Procedure:**
  - Three PCR tubes for each sample [for six Samples] were taken.
  - Tube 1 was for Genotype G set-I
  - Tube 2 was for Genotype G set-II
  - Tube 3 was for Genotype P
  - 10µl of RedDye PCR Master Mix was added in all 3 tubes.
  - 4µl of Genotype Primer Mix was added in all 3 tubes.
  - 9µl of Water was added in all 3 tubes.

- 2µl of cDNA was added in all 3 tubes.
- Finally Total reaction volume in all 3 tubes was about 25µl.
- PCR vials was Centrifuged briefly.

- PCR vials were placed into thermo cycler and Amplification Protocol programmed it as follows:
  - First step was Taq enzyme activation for 5min at temperature of 95ºC.
  - Second step was Denaturation for 30sec at temperature of 95ºC.
  - Third step was Annealing for 30sec at temperature of 58ºC.
  - Fourth step was Extension for 45sec at temperature of 72ºC.
  - Fifth step was Final extension for 5min at temperature of 72ºC.
  - Denaturation, Annealing & Extension were set for 35 cycles.

e) Gel electrophoresis:

Prepare 2% agarose gel as per standard procedure. Loaded entire PCR amplified product along with 10µl of 100bp DNA Ladder. Run electrophoresis and visualize in UV Transilluminator.

- **Procedure of Agarose gel electrophoresis:**
  - 2% agarose was prepared by mixing 2 gram of agarose in 100ml of 1X TAE buffer and melted using micro oven.
  - At 60ºC of agarose gel temperature, 5µl of Ethidium bromide was added.
  - Agarose solution poured slowly into the gel platform.
  - Gel was then kept undisturbed for to set, till the agarose solidifies.
  - Into the submarine gel tank 1XTAE buffer was poured.
  - Into the gel tank the gel platform was placed. Maintained the tank buffer level 0.5cm above than the gel.

- PCR Samples and 10µl of HELINI 100bp DNA Ladder are loaded after mixed with gel loading dye.
- Run electrophoresis at 50V till the dye reaches three fourth distance of the gel.
- Gel viewed in UV Transilluminator and observed the bands pattern.

4.8 STATISTICAL ANALYSIS

All statistical analysis was performed using SPSS version 22 with $p<0.05$ accepted as statistically significant. The Chi-square test was used for categorical variables, and ANOVA-test was used for continuous variables. (Refer APPENDIX 4)

CHAPTER 5: RESULTS

5.1 ANALYSIS OF CASES

During the study period from July 2014 to June 2015, a total number of 80 stool samples were collected from 80 children less than 5 years admitted in the pediatric diarrhoeal ward in Raja Mirasudhar Hospital, Thanjavur Medical College, Thanjavur. Samples were processed in the 24 hours Microbiology Diagnostic Laboratory, Thanjavur Medical College Hospital.

Table 5-1: Age and Sex Distribution

Age in months	**No of Cases**		**Sex Distribution**			
	No of cases	**No of cases (%)**	**Male**	**%**	**Female**	**%**
Less than 6 months	13	16%	8	62%	5	38%
7-12 months	29	36%	17	59%	12	41%
13-24 months	15	19%	10	67%	5	33%
25-36 months	15	19%	7	47%	8	53%
37-59 months	8	10%	4	50%	4	50%
Total	**80**	**100%**	**46**		**34**	

Table 5-1 shows that age and sex Distribution of 80 AGE cases reported. Majority of children were in the groups of 7-12 months (36%), followed by in age group of 13-24 months (19%), age group of 25-36 months (19%), age group of less than 6months is (13%) and least number of cases were reported in the age group of 37-59 months.

Figure 5-1: Age and Sex Distribution

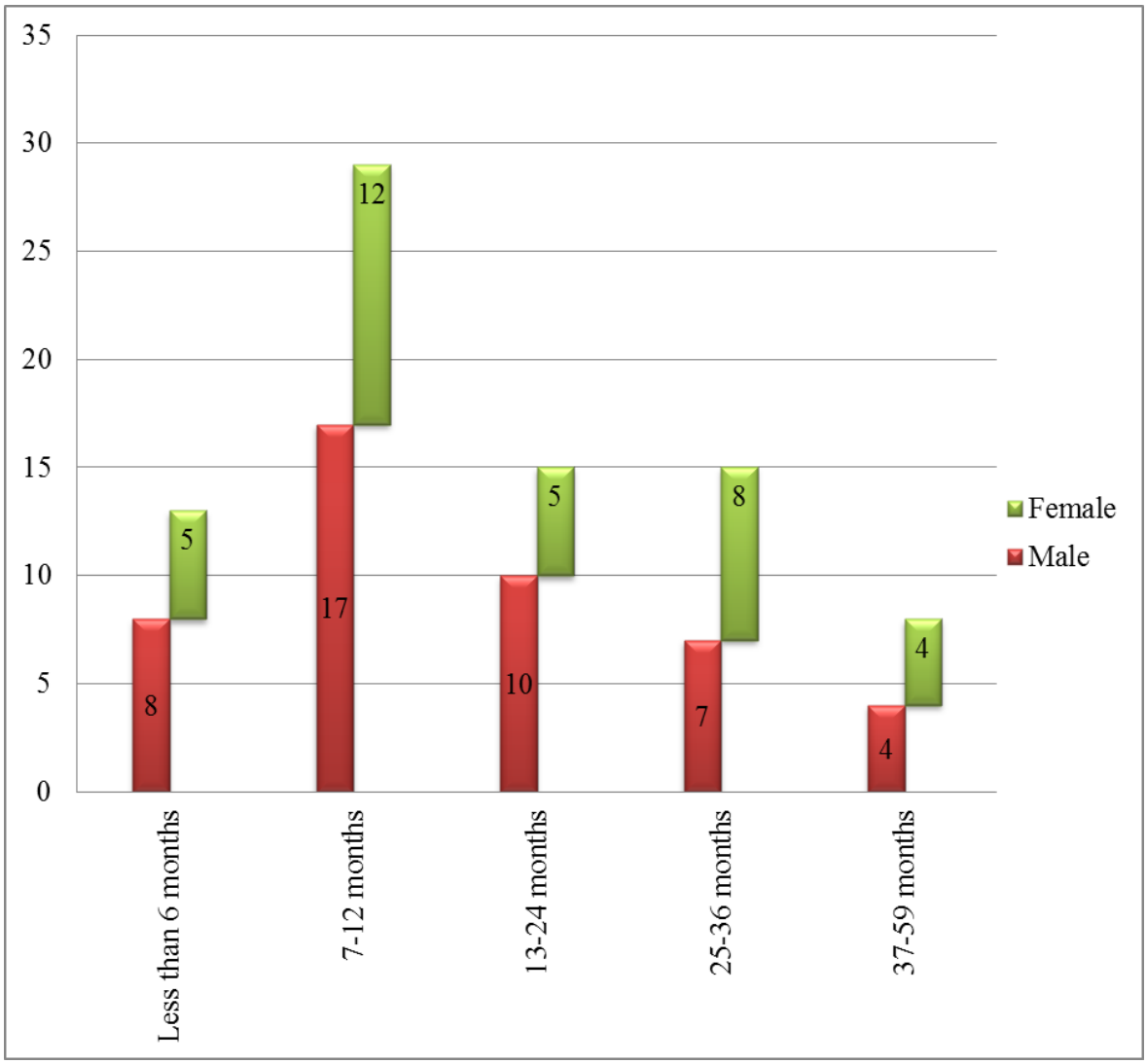
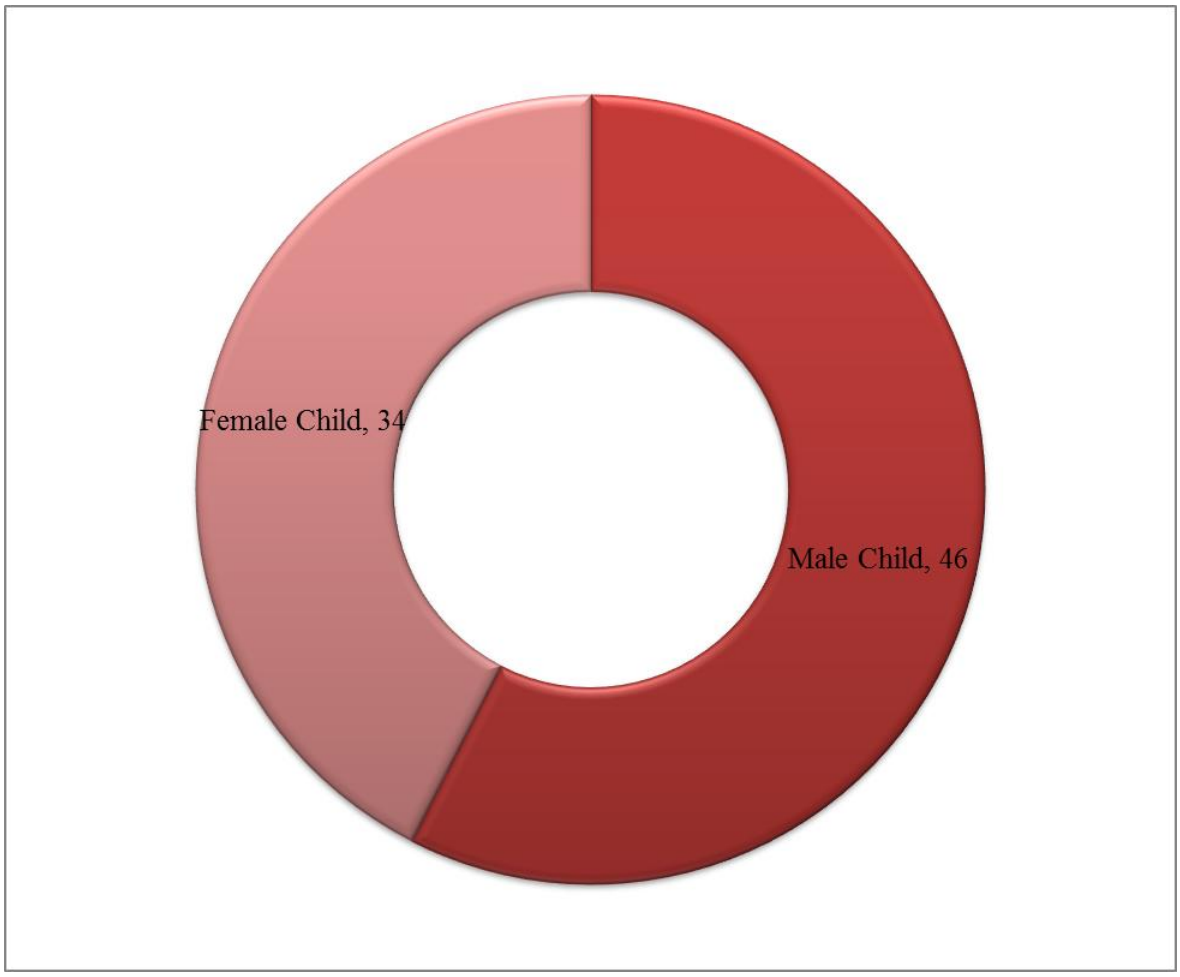


Figure 5-2: Sex Distribution



Out of total 80 children reported in this study, 46 (57%) were male children and 34 (43%) were female children. There were more male children than female children. All the 80 cases were from lower socioeconomic status only.

5.2 RESULTS OF ROTAVIRUS ANTIGEN ELISA TEST

Table 5-2: Rotavirus antigen positive by ELISA

Result	Total	%
Positive	23	29%
Negative	57	71%
Total	**80**	**100%**

Figure 5-3: Rotavirus Antigen Positive by ELISA

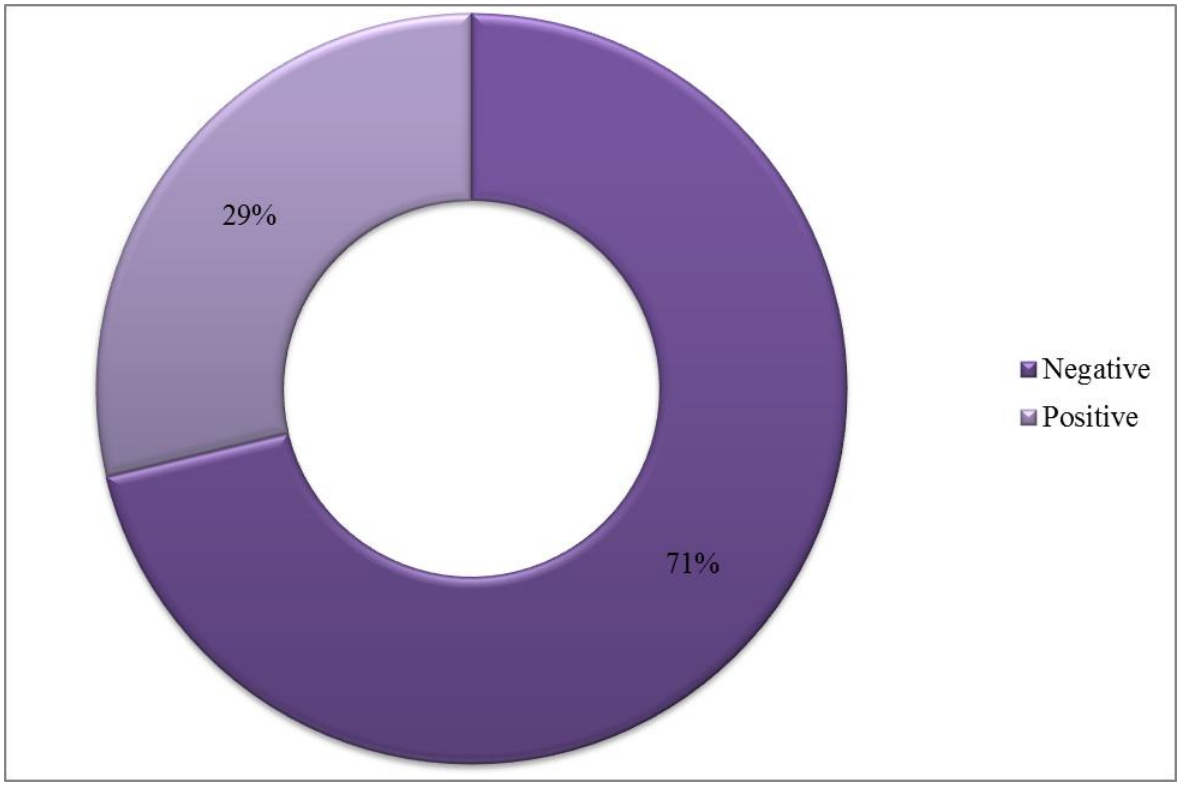


Table 5-2 and ***Figure 5-4*** the result of Rotavirus antigen detection by Rota clone ELISA kit that was tested for all 80 cases reported with AGE. Of which 23 (29%) were positive for Rotavirus antigen by Rotaclone ELISA method. Criteria for positive interpretation was colour of sample wells, were more intense than that of the negative control well was considered positive and absorbance units (A_{450}) by ELISA reader greater than 0.150 are considered positive. Remaining 57 (71%) were negative for Rotavirus antigen. Criteria for negative interpretation were colour of sample wells was equal to or less intense than the negative control well and absorbance units (A_{450}) by ELISA reader equal to or lesser than 0.150 are considered as negative.

Table 5-3: Age Distribution of RV Positive Cases

	Age in months	**No. of cases**	**No. of RV positive cases**	**% of RV positive cases for particular age group**
Group 1	Less Than 2 years	57	20	35%
Group 2	Older Than 2 years	23	3	13%
	Total	**80**	**23**	**29%**

Figure 5-4: Images of ELISA

Figure 5-4 shows pictures during the procedure of Rotavirus antigen detection by ELISA. First picture shows the microtitre plate for visual determination, second picture shows the microtitre plate which was tested for spectrophotometric determination. Third picture shows microplate reader with absorbance values.

Figure 5-5: Age Distribution

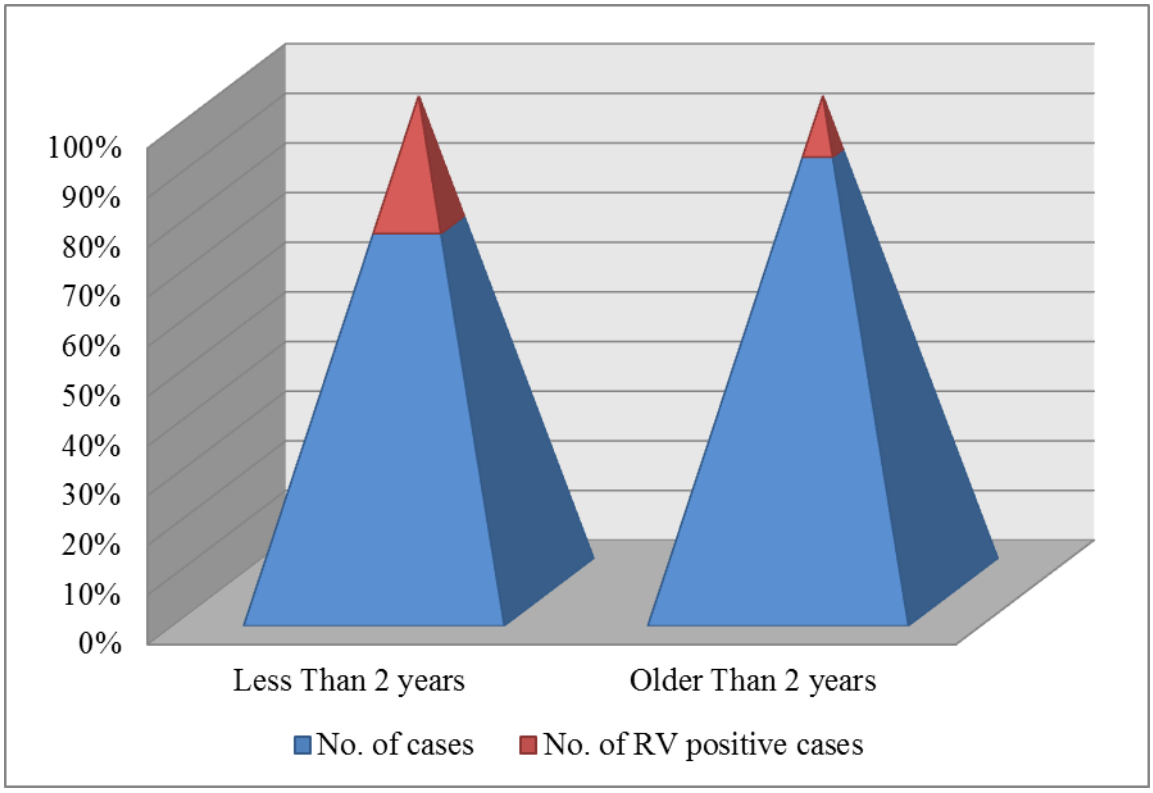


Table 5-3 and Figure 5-5 show the rotaviral antigen positivity with relates to age. For analysis age groups are dividing into two groups, group 1 and group 2. All children below 2 years are included in group 1 & all children above 2 years are included in group 2.

In group 1, out of 57 cases 20 are positive for Rotavirus antigen by ELISA test. Positivity rate in this group is 35%. In group 2, out of 23 cases, 3 are positive for ELISA Rotavirus antigen test. Positivity rate in this group is 13%.

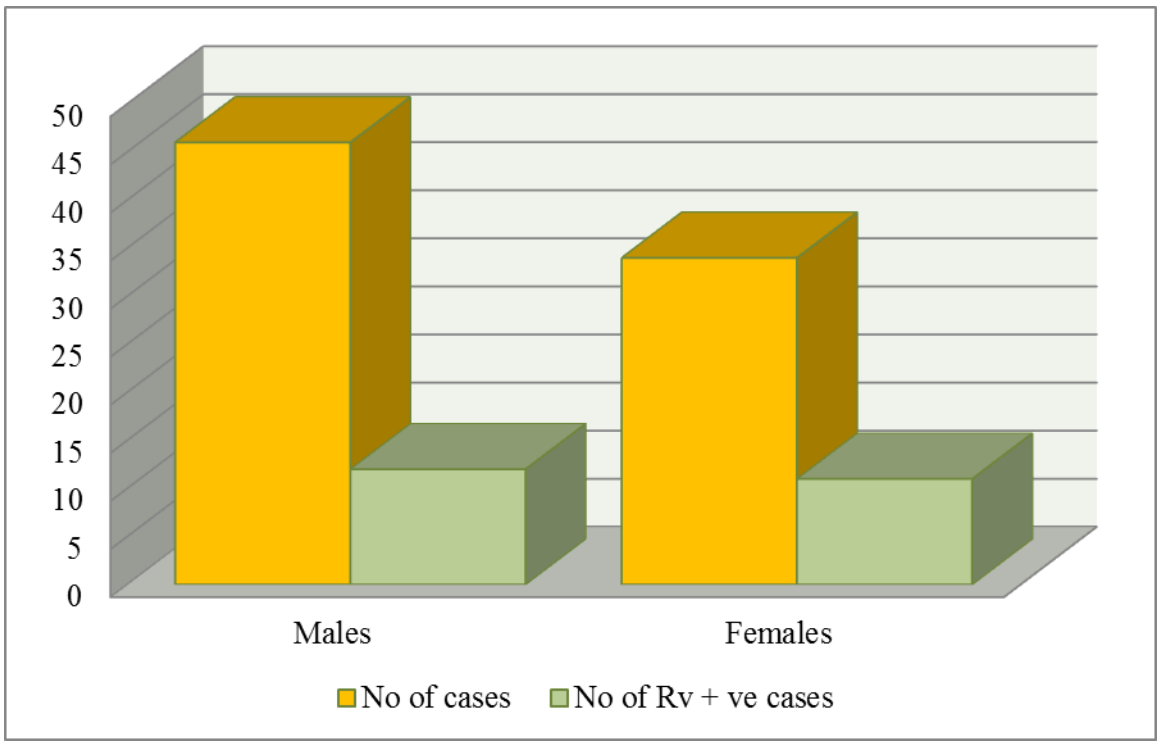
Statistical comparison of age factor in Rotavirus antigen positive and negative was done with chi-square test. Which shows p value of 0.04 (i.e.) < 0.05 %.

Table 5-4: Sex Distribution of RV Positive Cases

	Males	**Females**
No of cases	46	34
No of RV positive cases	12	11
% of RV positive cases	26%	32%
% of total no. of positive cases	15%	14%

Table *5-4* and Figure 5-6 shows sex distribution in Rotavirus positive cases. Out of 46 male children, 12 shows positive for Rotavirus antigen i.e.26% of male children are positive. Out of 34 female children, 11 were positive for Rotavirus antigen i.e. 32% female children are positive. Statistical comparison of sex distribution in Rota antigen positive and negative was done with chi square test. This test shows p value of 0.54 i.e. > 0.05.

Figure 5-6: Sex Distribution



5.3 SEASONAL ANALYSIS

Table 5-5: Seasonal Distribution of RV Positive Cases

Season	No. of cases	No of RV positive cases	% of RV positive cases
July to September	15	4	27%
October to December	30	10	33%
January to March	12	5	42%
April to June	23	4	17%
Total	***80***	***23***	***29%***

Figure 5-7: Seasonal Distribution

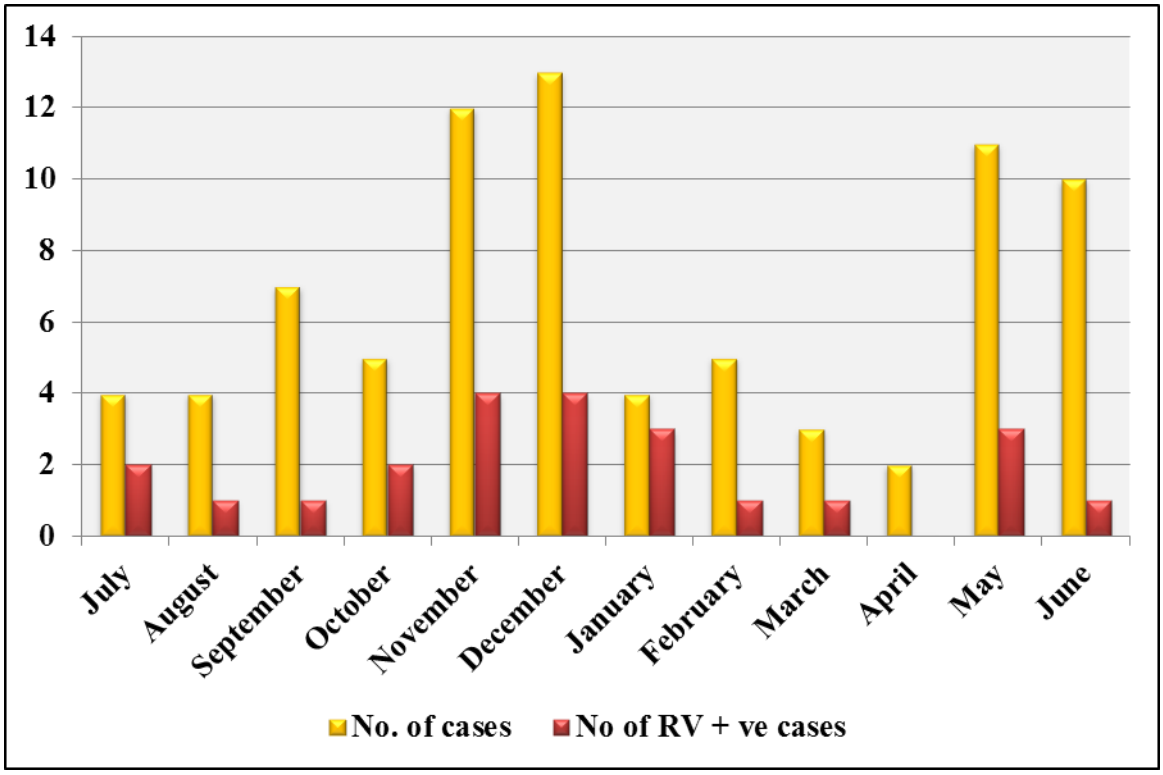


Table 5-5 and **Figure 5-7** shows seasonal distribution of RV positive cases from all 80 children under study. There were more cases positive during winter months such as October, November, December and January. Out of 23 positive cases 10 cases reported during October to December, 5 cases reported during April to June, 4 cases each reported during July to September and January to March.

To know the Significance of seasonal variation in Rotavirus positivity, ANOVA test was used. This method of statistics analysis gives the p value of 0.001, < 0.001

5.4 SYMPTOMS ANALYSIS IN ALL CASES

Table 5-6: Clinical Presentation

Symptoms	**No. of Cases**	**No. of RV positive cases**	**% RV positive Cases**
Diarrhoea	80	23	29%
Vomiting	51	19	37%
Fever	53	19	36%
↓ Urine Output	3	3	100%
Abdominal pain	4	2	50%
Septicemia	2	0	0%
Shock	1	0	0%
Respiratory infections	2	0	0%

Table 5-6 shows clinical presentation in all 80 cases as well as Rotavirus positive cases. Since in AGE the predominant symptom is diarrhoea all the 80 cases were presented

with diarrhoea. Fever was presented by 53 cases from total 80 cases and in 19 cases of 23 Rotavirus positive cases. Vomiting was seen in 51 of total cases and in 19 Rotavirus positive cases. All the 3 cases from 80 cases reported with decreased urine output are positive for Rotavirus antigen. About 4 cases were presented with abdominal pain, in that 2 cases are positive for Rota antigen.

About 5 cases were presented with other symptoms such as septicemia, shock and respiratory infections, all those cases are negative for Rota antigen.

5.5 ANALYSIS OF DEHYRATION

Table 5-7: Dehydration Status

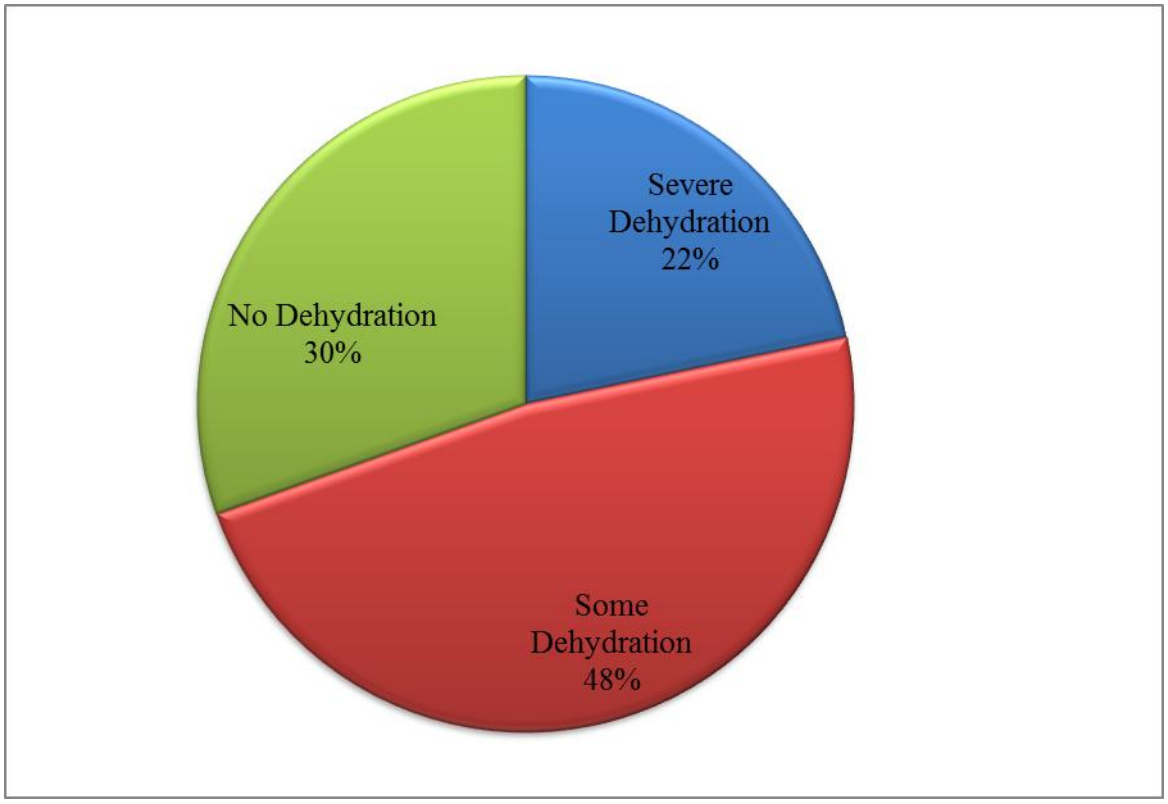
Dehydration	**No. of Cases**	**No. of RV positive cases**	**% RV positive Cases**
Severe	12	7	58%
Some	32	11	34%
No	36	5	14%
Total	***80***	***23***	***29%***

Table 5-7 shows dehydration status in Rotavirus antigen positive cases. Out of 12 cases reported with severe dehydration 7 are positive i.e. 58% of the cases reported with

severe dehydration are positive for Rotavirus antigen. Out of 32 cases reported with some dehydration 11 are positive i.e. 34% of the cases reported with some dehydration are positive for Rotavirus antigen. 5 cases are positive from 36 cases of without dehydration, i.e. only 14 % of the cases reported with no dehydration are positive for Rotavirus antigen.

Comparison of Dehydration status in those groups between positive and negative population was studied using chi square statistical analysis which results of p value of 0.003 i.e. < 0.05.

Figure 5-8: Dehydration Status



5.6 ANALYSIS OF HOSPITALIZATION

Table 5-8: Duration of Stay

Duration	**No. of Cases**	**No. of RV positive cases**	**% RV positive Cases**
Less than 2 days	27	0	0%
From 3 to 6 days	46	16	35%
Above 7 days	7	7	100%
Total	***80***	***23***	***29%***

Figure 5-9: Duration of Stay

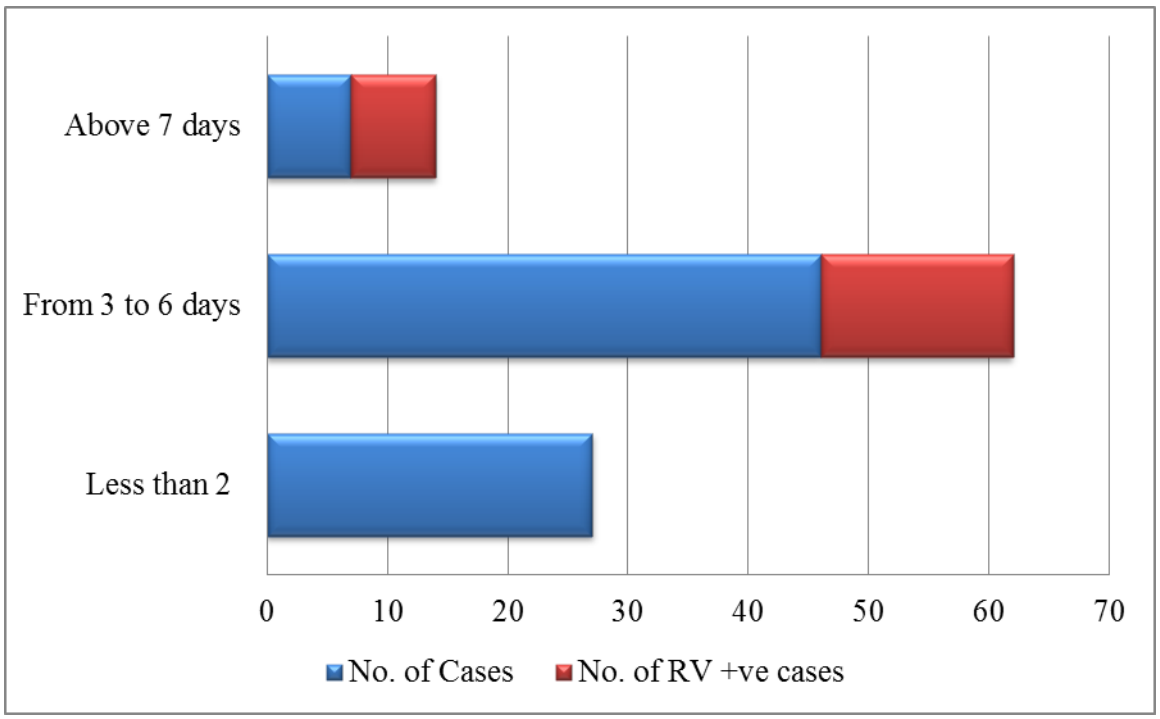


Table 5-8 and **Figure 5-9** shows the duration of hospitalization of all the 80 children under study. Inference from this table indirectly states that severity of acute gastroenteritis in Rotavirus positive cases than Rotavirus negative cases. All the 7 cases who are all admitted for more than seven days in hospital were positive for Rotavirus antigen. So, in this study all those who stayed longer than 7 days show 100% positivity for Rotavirus antigen. Out of 46 cases who are all admitted for 3-6 days in the hospital, 16 were positive for Rotavirus antigen. These shows about 35% of cases are positive for Rotavirus antigen. There were no positive cases reported in the population who stayed for less than 2 days i.e. 0% of positive cases seen in this group. Statistical analysis was done using chi square test, which results the p value of 0.0001, i.e. < 0.001.

5.7 RESULT OF BACTERIOLOGICAL EXAMINATION

Table 5-9 gives about the details of detection of bacterial isolates in all 80 samples. Totally 14 bacterial isolates are isolated from 80 samples. Out of 14 bacterial isolates, 2 isolates are seen in Rotavirus positive cases and 12 isolates are seen in Rotavirus negative cases. About 11 (78%) of all the bacterial isolates were *Escherichia coli,* about 2 (14%) were *Klebsiella pneumonia* and in one case (7%) *Shigella sp* was isolated. Two isolates reported in Rotavirus positive cases were *Escherichia coli.*

In this study two cases with septicemia and one case with shock were reported. All these 3 cases were below 1 year of age. Bacterial isolates in all 3 cases was *Escherichia coli.* All those cases are negative for Rotavirus antigen.

Table 5-9: Bacterial Isolates

Test Result	No. Bacterial isolates
RV positive	2
RV negative	12
Total	14

Bacterial isolates	No of cases
E. coli	11 (79%)
Klebsiella	2 (14%)
Shigella sp	1 (7%)
Total	***14***

5.8 RESULTS OF WET MOUNT OF STOOL SAMPLES

About 4 cases from 80 cases were positive for parasitic infestations. All those 4 children were above 3 years of age. Out of 4 cases, 3 cases positive for *Entamoeba histolytica* and in one case the parasite was found to be *Hymenolepsis nana*. All those 4 cases were negative for Rotavirus antigen.

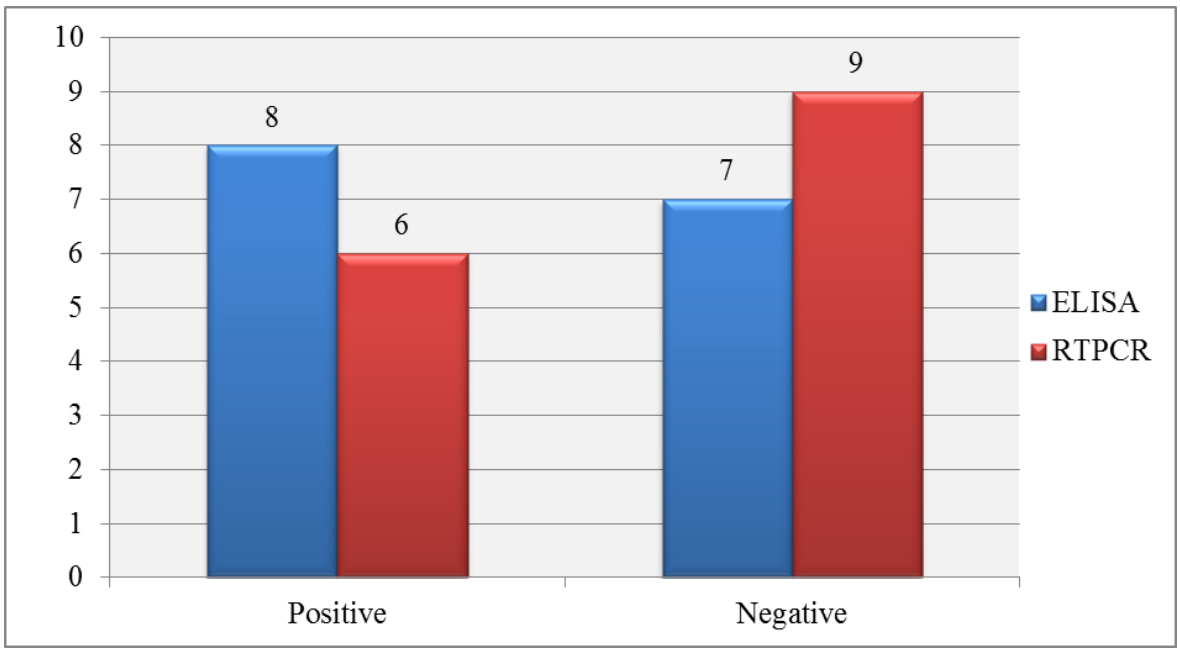
5.9 RESULT OF ROTAVIRUS-A REAL-TIME PCR ASSAY

Rotavirus-A Real-time PCR assay was done in randomly selected 15 stool samples including positive & negative samples by ELISA from 80 study groups.

Table 5-10: Results of RT PCR

Method	Positive	Negative	Total
ELISA	8	7	15
RT PCR	6	9	15

Figure 5-10: Results of RT PCR

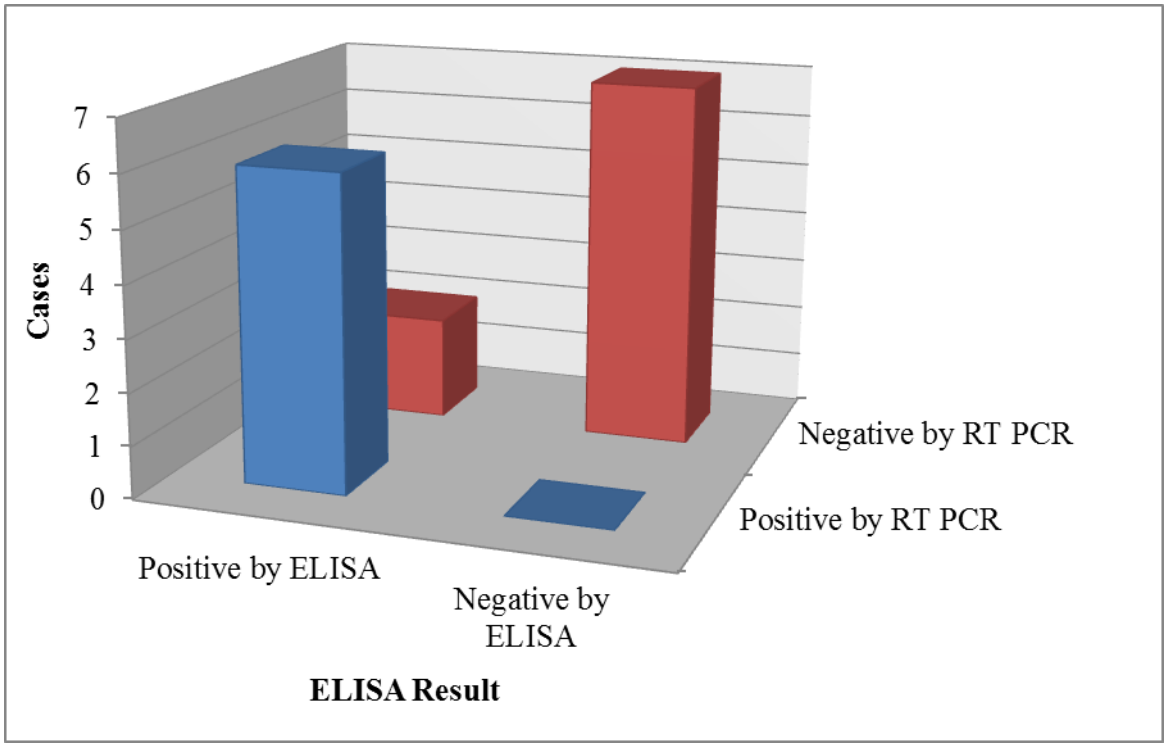


Out of 15 samples, 6 samples (40%) were positive and 9 samples (60%) were negative by Rotavirus-A Real-time PCR assay.

Table 5-11: Comparison of RT PCR and ELISA

	Positive by ELISA	Negative by ELISA	Marginal Row Totals
Positive by RT PCR	6	0	6
Negative by RT PCR	2	7	9
Marginal Column Totals	8	7	15 (Grand Total)

Figure 5-11: Comparison of RT PCR and ELISA



For randomly selected 15 samples of RVGE for children below 5 years from the study group, both the diagnostic tests were done. Out of 15 samples, 6 samples (40%) were positive by both ELISA & Rotavirus-A Real-time PCR. 7 samples (46%) were negative by both ELISA & Rotavirus-A Real-time PCR. Two samples (13%) which were positive by

ELISA were negative by RT PCR. The p value was calculated by using Chi-square test. The p value is 0.003(< 0.05).

5.10 QUANTITATIVE ANALYSIS BY rRT PCR

For all the six samples quantification of the viral load also elicited by rRT PCR. Viral load is determined by total number of viral copies per 200 mg of stool. Viral load depends upon duration of illness when the sample was collected. Among the six samples highest viral load seen in one of the samples was about 34,800 viral copies per 200 mg of stool, lowest viral load was about 1,050 viral copies per 200 mg of stool.

Table 5-12: Viral Load

Samples	**VIRAL LOAD** (Viral copies / 200 mg of stool)
Sample 1	3550 (3.550e+002)
Sample 2	1480 (1.482e+002
Sample 3	11250 (1.125e+003)
Sample 4	34800 (3.484e+003)
Sample 5	1050 (1.058e+002)
Sample 6	7175 (7.175e+002)

Figure 5-12: Plate Setup

MxPro - Mx3000P

Multiplex Quantitative PCR Systems
Quantitative PCR - Consolidated Report
C:\HELINI\2015\Rotavirus-A\18-09-2015.mxp
Filter gain factors: CY5 x1 ROX x1 HEX-JOE x1 FAM x8
Run date: September 18, 2015

Replicates: Treated individually (since no replicates in selection)

Plate Setup

	1	2	3	4	5	6	7	8	9	10	11	12
A		NTC FAM		NTC FAM		Unknown FAM		Standard 5.00e+005				
B		Unknown FAM		Unknown FAM		Unknown FAM		Standard 5.00e+004				
C		Unknown FAM		Unknown FAM		Unknown FAM		Standard 5.00e+003				
D				Unknown FAM		Unknown FAM		Standard 5.00e+002				
E				Unknown FAM		Unknown FAM						
F				Unknown FAM		Unknown FAM						
G				Unknown FAM		Unknown FAM						
H				Unknown FAM		Unknown FAM						

Figure 5-12 shows that plate setup of PCR vials in Thermocycler. A4 well for NTC (no template control). A8, B8, C8 & D8 for standards. Remaining 15 wells for samples (unknown).

Figure 5-13: Thermal Profile

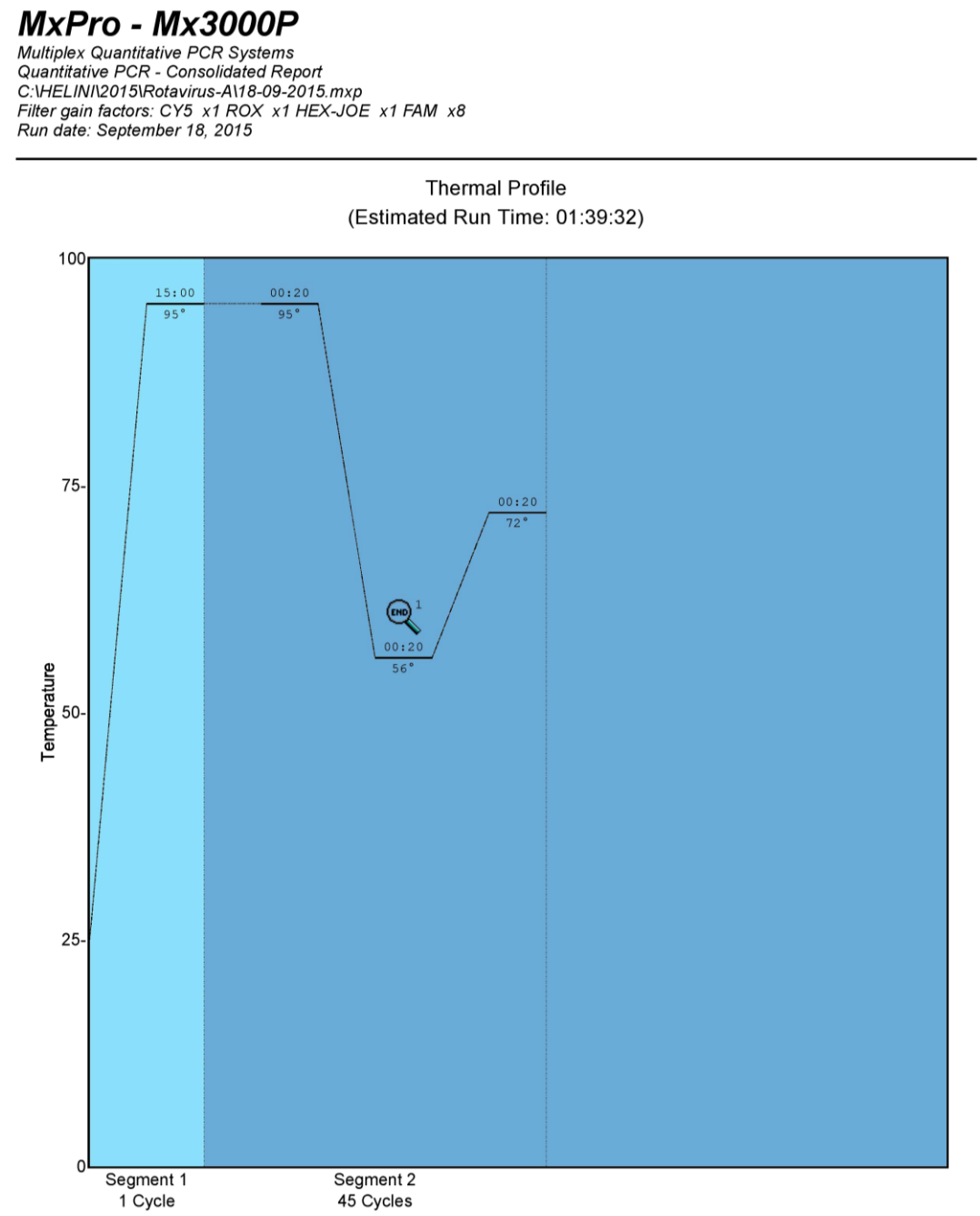


Figure 5-13 shows that thermal profile of the RT PCR procedure. Total estimate running time was about 1 hour 39 minutes 32 seconds. Segment 1 includes one step i.e. Taq enzyme activation its duration was about 15 min at temperature of 95ºC. Segment 2 includes 45 cycles of Denaturation, Annealing and Extension at 95ºC, 56ºC, 95ºC respectively. Duration was 20 sec for each step.

Figure 5-14: Amplification Plots

MxPro - Mx3000P

Multiplex Quantitative PCR Systems
Quantitative PCR - Consolidated Report
C:\HELINI\2015\Rotavirus-A\18-09-2015.mxp
Filter gain factors: CY5 x1 ROX x1 HEX-JOE x1 FAM x8
Run date: September 18, 2015

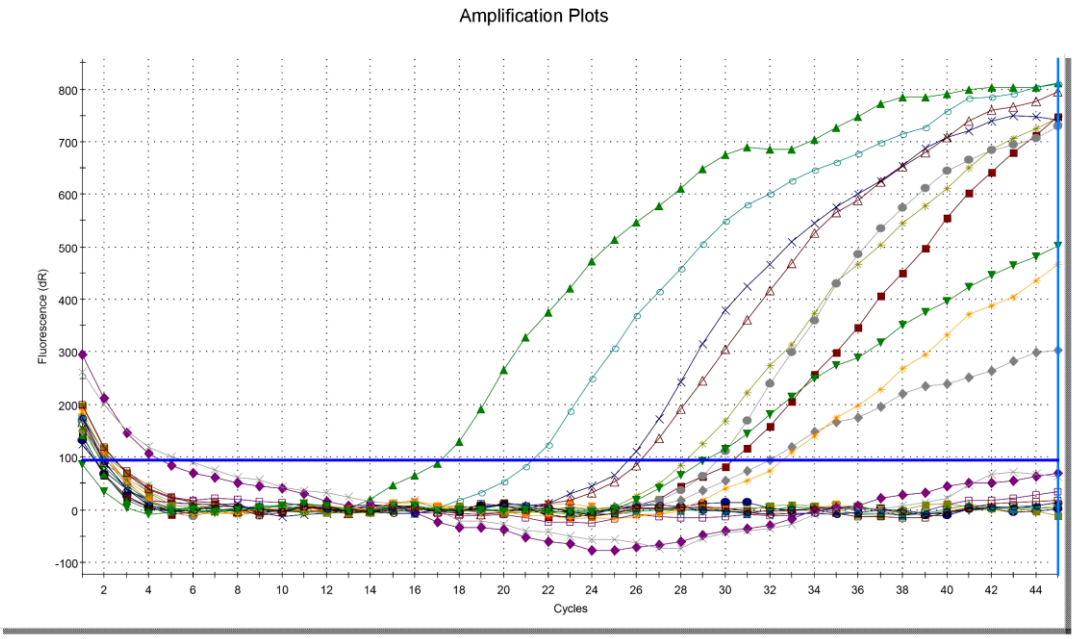


Figure 5-14 shows the amplification plots of both test samples and controls. The amplification plot is the plot of fluorescence signal versus cycle number. Amplification plots are created when the fluorescent signal from each 15 samples and 4 standards is plotted against cycle number. The threshold is shown by blue line, the level at which fluorescence signal shows statistically significant increase over baseline signal.

Figure 5-15: Standard Curve

MxPro - Mx3000P

Multiplex Quantitative PCR Systems
Quantitative PCR - Consolidated Report
C:\HELINI\2015\Rotavirus-A\18-09-2015.mxp
Filter gain factors: CY5 x1 ROX x1 HEX-JOE x1 FAM x8
Run date: September 18, 2015

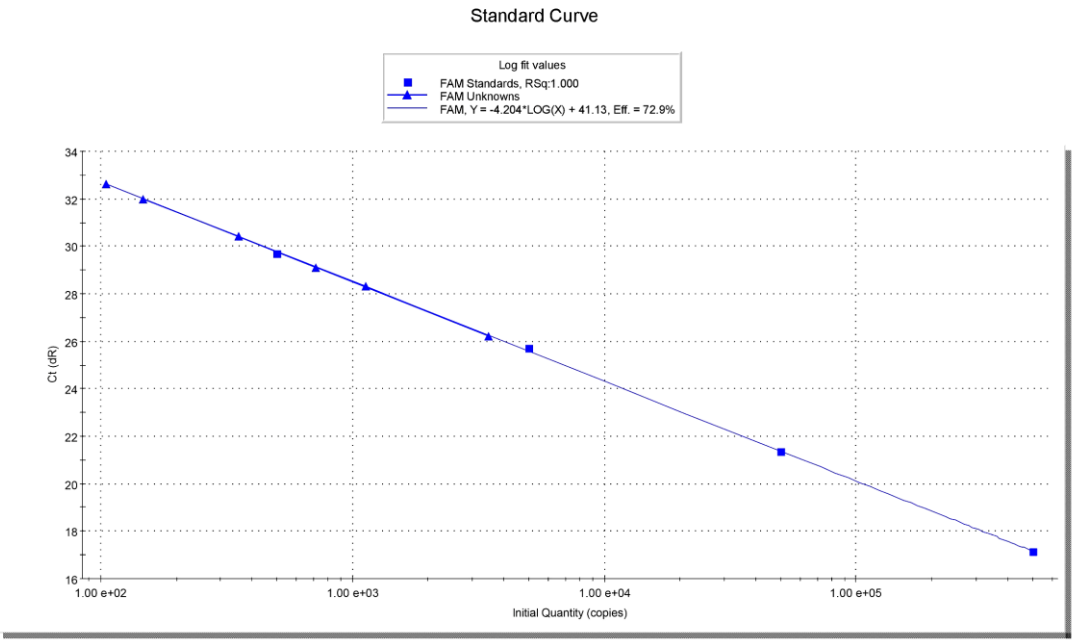


Figure 5-15 shows the standard curve of viral load of both test samples and standard curve shows threshold cycle (Ct) on the y-axis and the starting quantity of RNA target on the x-axis. The threshold cycle (Ct) is the cycle number at which the fluorescent signal of the reaction crosses the threshold. The Ct is used to calculate the initial RNA copy number, because the Ct value is inversely related to the starting amount of target. For six samples the Ct was 33rd, 32nd, 30th, 29th, 28th and 26th cycle respectively.

Figure 5-16: Text Report

MxPro - Mx3000P

Multiplex Quantitative PCR Systems
Quantitative PCR - Text report
C:\HELIN\2015\Rotavirus-A\18-09-2015.mxp
Filter gain factors: CY5 x1 ROX x1 HEX-JOE x1 FAM x8
Run date: September 18, 2015

Thermal Profile Summary

Segment	Cycles	Plateau	Temp. (degrees)	Temp. Inc. (deg/sec)	Duration (min:sec)	Time Inc. (min:sec)	Collect
1	1	Plateau 1	95.0	0.0	15:00	00:00	<none>
2	45	Plateau 1	95.0	0.0	00:20	00:00	<none>
2	45	Plateau 2	56.0	0.0	00:20	00:00	1 Endpoints
2	45	Plateau 3	72.0	0.0	00:20	00:00	<none>

Replicates: Treated individually (since no replicates in selection)
* Fluorescence term used: dR

Text Report

Well	Well Name	Well Comment	Dye	Well Type	Threshold*	Ct*	Quantity (copies	RSq*	Slope*
A4	10-10-1.5/ 5u	5ul Bio IC5ul Lam IC	FAM	NTC	93.910	No Ct	No Ct	1.000	-4.204
A6	8	---	FAM	Unknown	93.910	30.41	3.550e+002	1.000	-4.204
A8	---	---	FAM	Standard	93.910	17.15	5.00e+005	1.000	-4.204
B4	1	---	FAM	Unknown	93.910	32.00	1.482e+002	1.000	-4.204
B6	9	---	FAM	Unknown	93.910	28.30	1.125e+003	1.000	-4.204
B8	---	---	FAM	Standard	93.910	21.34	5.00e+004	1.000	-4.204
C4	2	---	FAM	Unknown	93.910	No Ct	No Ct	1.000	-4.204
C6	10	---	FAM	Unknown	93.910	No Ct	No Ct	1.000	-4.204
C8	---	---	FAM	Standard	93.910	25.70	5.00e+003	1.000	-4.204
D4	3	---	FAM	Unknown	93.910	26.24	3.484e+003	1.000	-4.204
D6	11	---	FAM	Unknown	93.910	No Ct	No Ct	1.000	-4.204
D8	---	---	FAM	Standard	93.910	29.71	5.00e+002	1.000	-4.204
E4	4	---	FAM	Unknown	93.910	No Ct	No Ct	1.000	-4.204
E6	12	---	FAM	Unknown	93.910	No Ct	No Ct	1.000	-4.204
F4	5	---	FAM	Unknown	93.910	No Ct	No Ct	1.000	-4.204
F6	13	---	FAM	Unknown	93.910	32.61	1.058e+002	1.000	-4.204
G4	6	---	FAM	Unknown	93.910	No Ct	No Ct	1.000	-4.204
G6	14	---	FAM	Unknown	93.910	No Ct	No Ct	1.000	-4.204
H4	7	---	FAM	Unknown	93.910	29.12	7.175e+002	1.000	-4.204
H6	15	---	FAM	Unknown	93.910	No Ct	No Ct	1.000	-4.204

Figure 5-16 shows text report of Real time PCR for RNA identification of Rotavirus in 15 samples along with 4 standards. Well A4 is for NTC i.e. no-template control. FAM channel fluorescence dye used for data collection. Well type denotes about well contains NTC, unknown is the test samples and standards. Threshold level is 93.910. Ct is cycle threshold, for four standards Ct was 17.15, 21.34, 25.70 and 29.71 respectively, for NTC and negative samples there was no Ct. Quantity of viral load was depicted by number of copies per 200 mg of stool. Rsq is R squared i.e. Correlation coefficient (R2) is a measure of how well the data fit the standard curve, R2 is 1.000 which is ideal. The slope of the log-linear phase of the amplification reaction is a measure of reaction efficiency. Here the slope value was -4.204.

5.11 RESULT OF MOLECULAR CHARACTERIZATION BY GENOTYPING

Six samples which were positive by Rotavirus-A Real-time PCR assay was further processed for Molecular characterization by Genotyping. Conventional Polymerase Chain Reaction was done for 6 samples for the G (VP7) genotyping and P (VP4) genotyping detection. Out of six samples, G genotypes of 3 samples were **G1 (50%)**, for remaining 3 samples G genotypes were **G2** (50%). Out of six samples P genotypes of 3 samples were **P[4] (50%)**, P genotypes of 2 samples were P[8] **(33%)**, and for one sample resulted as partial genotyping (17%) due to there was no amplification during P genotyping.

G-P type combination most frequently found in this study was G2P[4] (50%) followed by G1 P[8] (33%). Remaining 17% was untypable.

Table 5-13: Rotavirus genotypes and their base pair ranges

Sample no	G genotyping	Base pair range	P genotyping	Basepair range	G-P type combinations
1	G1	215bp	P8	250bp	G1P[8]
2	G2	351bp	P4	369bp	G2P[4]
3	G2	351bp	P4	369bp	G2P[4]
4	G1	215bp	No amplification	-	G1
5	G2	351bp	P4	369bp	G2P[4]
6	G1	215bp	P8	250bp	G1P[8]

Figure 5-17: Distribution of Rotavirus G-P Genotypes:

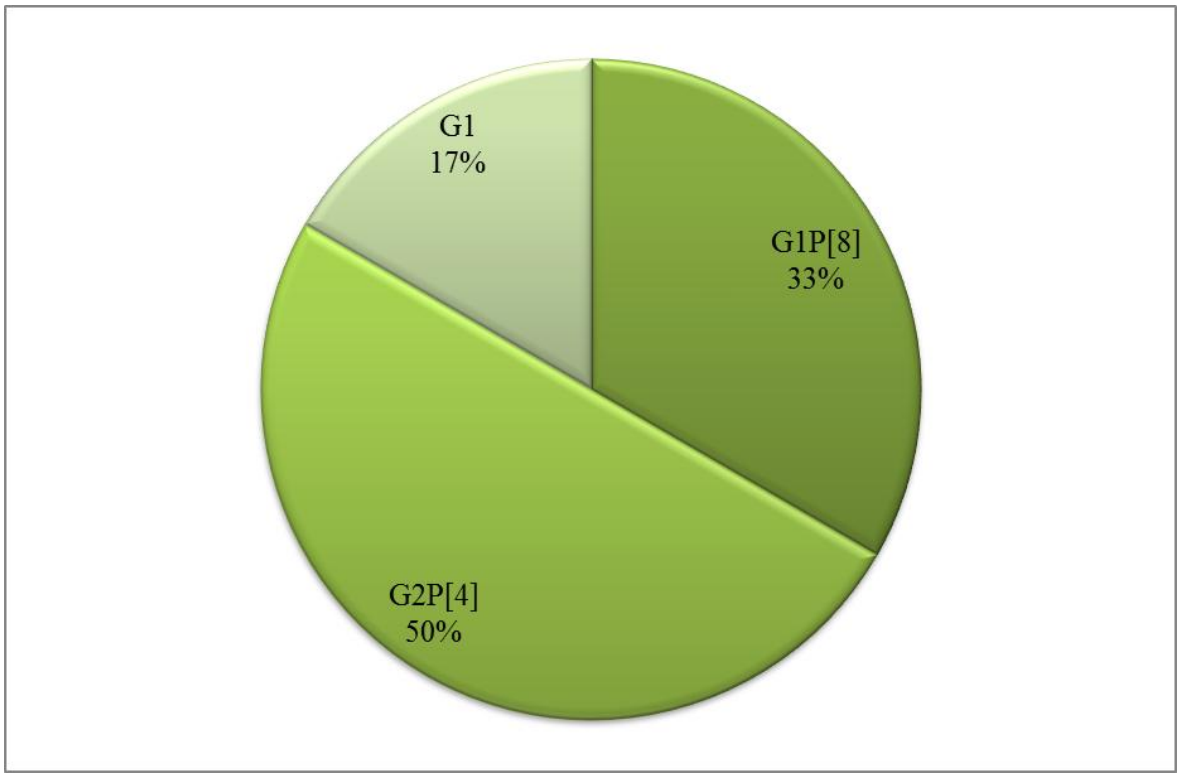


Figure 5-17 shows Distribution of Rotavirus Genotypes in six samples. Commonest G-P combination among the six samples was G2P[4], followed by G1P[8], for one sample there was partial genotyping.

Age parameters, seasonal parameters and clinical parameters were analysed for all those six samples. There was no significance difference between G2P[4] &G1P[8] in all those parameters. They show equal characteristics among them.

Figure 5-18: G Typing

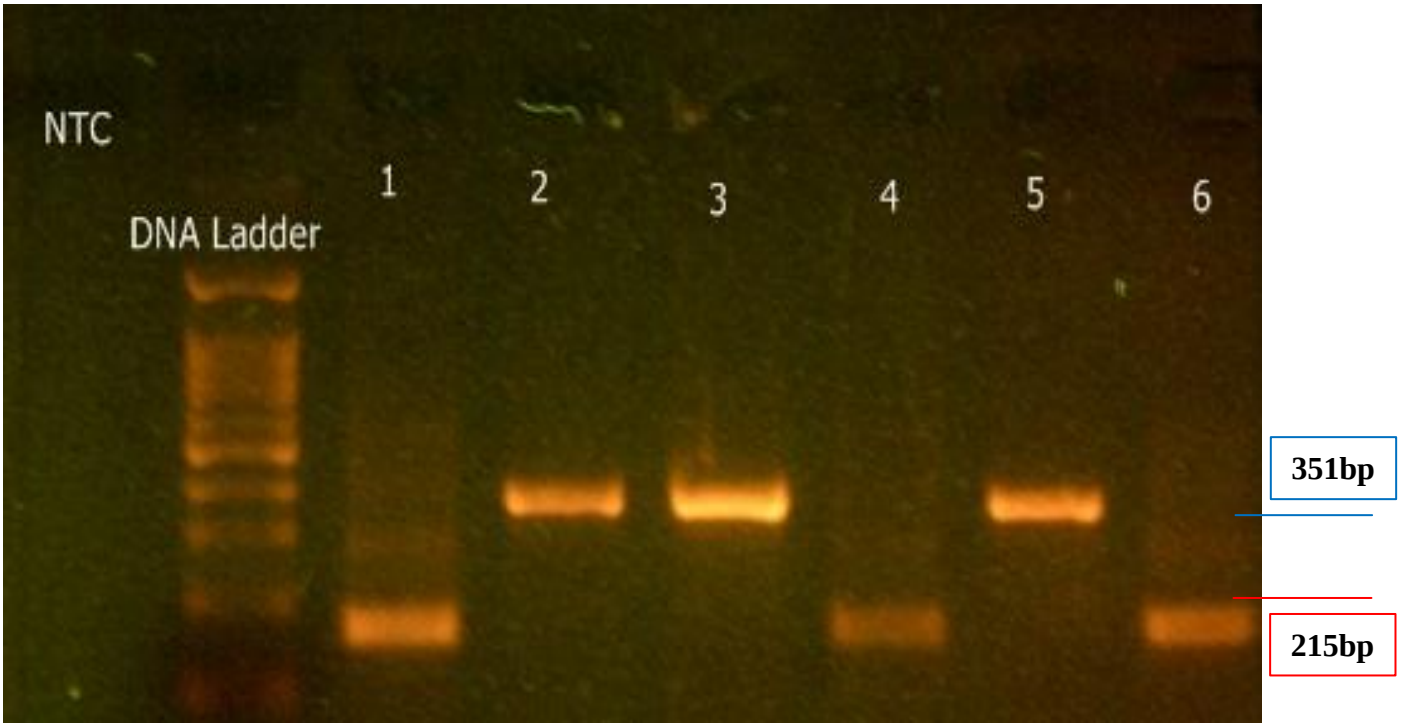


Figure 5-18 of Rotavirus-A G (VP7) Genotyping shows the picture of Gel viewed in UV Transilluminator after Agarose gel electrophoresis to observe pattern of the bands in conventional PCR method. NTC was no template control, DNA Ladder of 100bp, samples 1-6. Base pair range of samples 1, 4 and 6 were 215 bp which was typed as G1. Base pair range of samples 2, 3 and 5 were 315 bp which was typed as G2.

Figure 5-19: P Typing

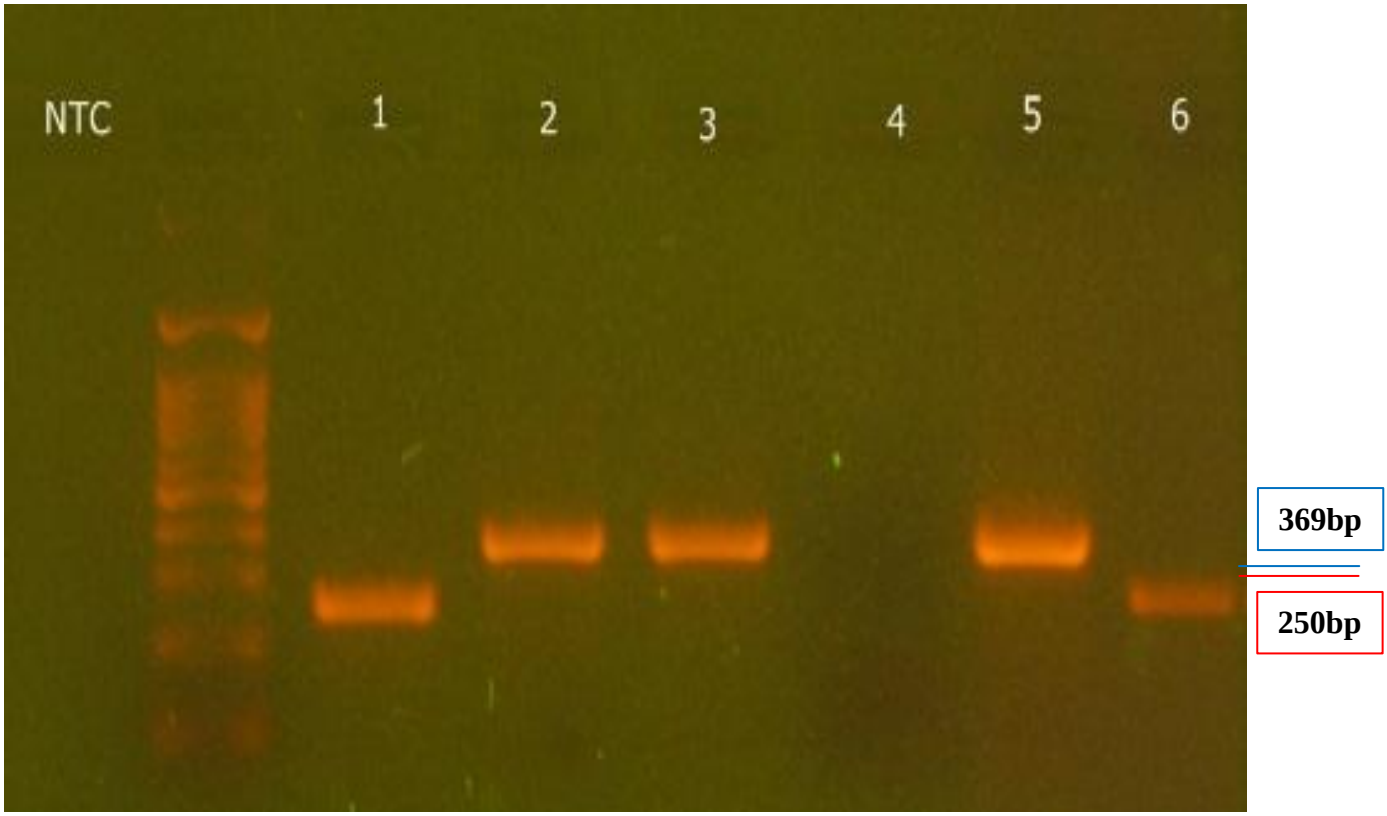


Figure 5-19 of Rotavirus-A P(VP4) Genotyping shows the picture of Gel viewed in UV Transilluminator after Agarose gel electrophoresis to observe pattern of the bands in conventional PCR method. NTC was no template control, DNA Ladder of 100bp, samples 1-6. Base pair range of samples 1 and 6 were 250 bp which was typed as P[8]. Base pair range of samples 2, 3 and 5 were 369 bp which was typed as P[4]. Sample 4 shows no band formation resulted as untypable.

CHAPTER 6: DISCUSSION

Rotavirus is the leading common cause of severe, life threatening gastroenteritis in children less than five years. Acute diarrhoel diseases continue to be major cause of morbidity and mortality in children less than 5 years in developing countries like India. Rotavirus is the cause for 23-39% of hospitalized diarrhoea in children. Rotavirus is the cause for about 39% diarrhoeal mortality in children less than 5 years. More than 80% of RVGE cases were under two years of age.[2 3 5] Rapid diagnostic procedures like Rota antigen detection by ELISA provides usual information to the physician, may prevent inappropriate treatment and nosocomial infections by intervening the spread of the infections in hospital set up.

This study was done to determine the etiological factors for acute diarrhoea in children less than five years in tertiary hospital, Thanjavur. About 80 children who are all diagnosed as having acute gastro enteritis and admitted in the pediatric ward were included in this study. This study was done over a period of one year from July 2014 to June 2015. In this study youngest child was one month old and oldest child was 4 years 10 months old.

6.1 PREVALENCE OF ROTAVIRUS

In this study the prevalence of Rotavirus was 29%. This value almost rationale with the prevalence rates of rotaviral infections in developing countries. The global prevalence of Rota virus is 23-39% of hospitalized diarrhoea in children; in this present study also the prevalence of RVGE is in line with global prevalence.

This prevalence rate is similar to prevalence rate (28.57%) of the study by Shetty et al., during 2014 at Karnataka.[21] Manohar et al., states that 25.66% of the children aged under 5 years of diarrhoea are due to Rotavirus.[90]

In another study by Sanjay C Chavan from western Maharashtra reported 38% of prevalence during 2010.[7]

In 2011, Mathew from Kerala reported 35% of prevalence.[91] Babji.S from Nagercoil reported high prevalence rate of 51% during 2010.[6] In another study by Shariff M from Nepal reported 35% of prevalence of rotaviral infections in children during the period in 2003.[92]

In 2001, Zafer Kurugol from turkey reported about 39% of prevalence of Rotavirus.[5] Kafi SK from Sudan reported 33% of prevalence of Rotavirus in under five children.[93] In India high prevalence were reported from Kerala (70%) and Manipur (89%) due to epidemiological nature of these places.

6.2 AGE PREVALENCE

Out of 80 children in this study, majority of children belongs to 7-12 months of age (36%). In **Table 5-2** out of 23 (29%) positive children for Rotavirus antigen, 20 (35%) were in the age group below 2 years and 3 (13%) were in the age group above 2 years. There was a significant difference between the age group of less than 2 years and age group of more than 2 years($p<0.05$). Rotavirus primarily affects less than 2 years;[23] in this present study also the age prevalence is in line with global age prevalence of Rotaviral infections.

Under five age distribution in positive children in this study is similar to other studies in the world wide by Zafer Kurugol et al.,[5] Sanjay C Chavan et al.,[7] shetty et al.,[21]. Mathew et al.,[91] Shariff M et al.,[92] Kafi SK et al.,[93] According to WHO Scientific working group 6-

24 months of age is vulnerable group for RV infections, which was observed in this study also.

6.3 SEX PREVALENCE

In the present study out of 80 children, 46 (58%) were male children and 34 (42%) were female children. Out of 23 (29%) positive children, 12 (26%) were male children and 11 (32%) were female children. Sex distribution in Rotavirus positive and negative was almost similar. There was no significant difference between the sexes in this study ($p>0.05$). Zafer Kurugol et al., stated that prevalence in both male and female children was same.[5] Sanjay C Chavan et al., also stated sex prevalence of Rota positive cases were same in both sexes,[7] both the above studies were similar to the present study in the aspect of sex prevalence in rotaviral infections. Kafi SK et al., stated that prevalence in male children was higher than female children.[93] Shetty et al., also stated that higher prevalence in male children.[21] Results in these studies were not observed in the present study.

6.4 SEASONAL PREVALENCE

Seasonal distribution in all 80 cases as well as positive and negative cases in this study clearly states that Rotavirus infection occurs throughout the year but peak infection occurs during winter months November to January. Out of 30 cases, 10 were positive during October to December about 33% of prevalence during these months. Out of 12 cases, 5 cases were positive during Jan - March shows prevalence of 42%. Totally there was high prevalence rate during winter months from October to March. There was significant difference of Rotavirus prevalence between winter months and other seasons ($p<0.001$). This specific seasonal distribution of Rotaviral infections may due to low humidity which facilitates the survival of the virus in surfaces.[23]

In a previous studies by Zafer Kurugol et al. & Sanjay C Chavan et al., RGE was more prevalent in winter months and its similar to our results.[5 7]

In a study by Sheriff et al., Rotavirus infection was appear throughout the year but peak in late winter.[21] Kafi SK et al., also states that Rotavirus infection occurred throughout the year but peak in winter.[93] All those above studies are similar to the present study for the seasonal prevalence of Rota infections in children. In a study by Mathew et al., RV infections were seen throughout the year, but most common during the hot dry months which were different from all other studies.[91] Findings from all the above studies imply that Rotaviral infections were seen throughout the year, seasonal variation depends upon the particular geographical area.

6.5 SYMPTOMATIC PREVALENCE

The clinical presentation in all 80 cases and Rota viral positive cases were showed in **Table 5-7**. Diarrhoea present in all cases in this study showed 100% prevalence. 87% of RV positive children presented with vomiting which was considered as next predominant symptom. So, prevalence of vomiting was 41%. Percentage of RV positive children presented with fever was about 74%. Prevalence rate for fever was 31%. Decreased urine output was present in 13% of RV positive cases, but prevalence rate was about 100%. Abdominal pains were present in 8% of RV positive cases, but the prevalence rate was about 50%. Many studies states that vomiting was significantly more frequently observed in rotavirus-infected children;[49] in this present study also vomiting was the predominant symptom next to diarrhoea.

In previous studies by Sanjay C Chavan et al., stated that diarrhoea and vomiting was predominant symptom in RVGE.[7] Shetty et al., stated that diarrhoea and vomiting was seen

in 100% of positive cases.[21] Both the above studies were similar to the present study regarding symptomatic prevalence.

6.6 PREVALENCE OF DEHYDRATION

Dehydration status was considered as important parameter for severity of RVGE children. **Table 5-7** shows the prevalence of Dehydration in all 80 cases and in 23 positive children. Out of 10 cases with severe Dehydration, 5 are positive for Rotavirus antigen i.e., prevalence of severe dehydration in Rotavirus infection in this study was 50%. Out of 32 cases with some dehydration, 11 were positive for Rotavirus antigen i.e, prevalence of some dehydration in Rotavirus infection in this study was 34%. So, out of 23 positive cases, 16 cases presented with dehydration i.e. percentage of dehydration in positive cases in this study was 69.5%. And the prevalence rate of dehydration in this study was about 38%. There was high significant difference between dehydration positive cases and without dehydration positive cases ($p < 0.05$). Since prevalence of dehydration was high in rotaviral infections; early diagnosis and prompt management of dehydration plays a vital role in preventing mortality.

Similarly, Sanjay C Chavan et al states that, percentage of dehydration in RV positive cases was 87%.[7] And Sheriff et al states that, percentage of dehydration in RV positive cases was about 76%.[92]

6.7 PREVALENCE OF DURATION OF HOSPITAL STAY

Duration of hospitalization indirectly implies about the severity of the disease in RVGE. ***Table 5-8*** shows distribution of cases regarding to duration of stay in the hospital in total 80 cases and 23 RV positive cases. All 7 cases from the total population in this study who stayed for more than seven days were positive for RVGE. This shows the prevalence of 100% in > 7 days of stay. Percentage of this group in 23 positive cases was

about 30%. Out of 43 children from total study group who stayed for 3-7 days of stay, 16 were positive for RVGE i.e. 37% of prevalence. Percentage of this group in 23 positive cases was about 70%. Out of 27 cases from total study group who stayed for less than 2 days none showed positive for Rotavirus antigen. This shows 0% prevalence in this group. There was high significant difference of Rotavirus prevalence in between long duration of hospitalization and short duration of hospitalization (p value <0.001).

In a previous study by Mathew et al., which stated that there was high significance between duration of stay in hospitalized children who are diagnosed as having Rotavirus gastro enteritis,[91] also, Saia et al., stated that RVGE gave considerable burden of hospital admissions by longer duration of hospitalization.[8] Statement by above studies by Mathew et al & Saia et al were similar to the present study regarding hospitalization.

6.8 PREVALENCE OF BACTERIAL CAUSES

Distribution of AGE due to bacterial causes was shown in **Table 5-9**. About 14 cases from 80 study population, bacteria were isolated from their stools. Prevalence of bacterial cause for diarrhoea in this study was about 17%. In 2 (8%) cases rotaviral antigen was detected along with bacterial isolates. Out of 14 bacterial isolates, 11 were *E. coli* (79%). This shows *E. coli* is another important etiological factor for acute diarrhoel disease in under five children.[20] The other bacterial isolates were *Klebsiella* and *Shigella sp* isolated in 2 cases and 1 case respectively. Similarly, Sheriff et al prevalence of bacterial cause was 16%, about 11% of Rota positive cases detected combination with bacterial cause. *E. coli* was predominant bacteria that were isolated (67%).[92] According to black et al Rotavirus and *E. coli* were commonest cause for diarrhoea in less than two years of age.[94] Above studies along with present study state that *E. coli* was another common cause for diarrhoea in children.

6.9 PREVALENCE OF PARASITIC INFECTION

Out of 80 acute gastro enteritis cases, 4 cases were diagnosed as having parasitic infections. The prevalence was about 5%. Out of 4 cases, 3 were detected as *Entaemoba sp* and one case was detected as *Hymenolepsis nana.*

In the previous study by Sheriff et al., which stated that prevalence of parasitic infection in under five children was about 6.25%. Commonest one being *Entaemoba histolytica*.[92] In another study by Bijay Ranjan Mirdha stated that commonest parasites that cause infections in children were *Entaemoeba sp, Hymenolepsis nana, Ascaris* & *Giardia*.[95]

6.10 DISCUSSION ON ROTAVIRUS-A REAL-TIME PCR ASSAY

For randomly selected 15 samples of RVGE for children below 5 years from the study group, both the diagnostic tests were done. Since both these tests were not done on control group i.e. on normal children, sensitivity and specificity of these tests cannot be compared. Out of 15 samples, 6 samples (40%) were positive by both ELISA and Rotavirus-A Real-time PCR. 7 samples (46%) were negative by both ELISA and Rotavirus-A Real-time PCR. Two samples (13%) which were positive by ELISA were negative by RT PCR. Samples which were positive initially by ELISA and negative later by RT PCR were considered as borderline positives. Reason for positivity in ELISA is due to presence of VP6 antigen, negativity in RT PCR may be due to low quantity of viral load may get degraded by time when it tested for molecular method.[6]

Among the six samples, highest viral load seen in one of the samples was about 34,800 viral copies per 200 mg of stool, lowest viral load was about 1,050 viral copies per 200 mg of stool. Viral load depends upon duration of illness when the sample was collected and also related to severity of the illness.

Comparison can be done in the basis of difference in significance between these two tests in diagnosing Rota viral infections. Chi-square test was used for to calculate significance value. The p value was 0.03 (< 0.05). There is significant difference between these two tests in diagnosing Rotavirus infections in stool samples. Comparatively RT PCR was more sensitive than ELISA in detecting true positive cases. But method of choice is ELISA due to its relatively lower cost, also it not require special instruments and easily available.

In a study by Iyoha and Abiodun (2014) states that most sensitive method in detecting Rotavirus in stool is RT PCR analysis when compared to ELISA, RT PCR may not readily available for routine diagnosis of Rotavirus in our laboratory due to high cost. Also Andi Yasmon et al states that about 3.7% cases were positive in ELISA were negative by RT PCR. This study supports the present study.[79] In a study by Catriona Logan et states that Real-time PCR resulted in 100% and 186% increases in the rate of detection of adenovirus and Rotavirus, respectively.[62] Another study by Cathy V. Knisley also states that, molecular method has more sensitivity and specificity, make it superior in its ability to detect Rotavirus.[67] Xiaoli L.Pang, et al states that real time RT-PCR assay is both simple and rapid with enhanced sensitivity and a lower risk for cross-contamination.[96] In a study by Buesa et al states that in their study 30% positive detection rate was observed by RT/PCR while 29% positive detection was by ELISA. RT/PCR was 100 times more sensitive than the ELISA test in detecting Rotaviruses.[97]

From the above studies and the present study it is clear that there is significant difference between ELISA and RT-PCR in diagnosing Rotavirus infections in stool samples.

6.11 PREVALENCE OF G-P GENOTYPES

For all those six positive RT PCR samples, G and P typing was carried out by amplification of variable sequences of the VP7 and the VP4 genes with eight G- and six –P type-specific primers (conventional multiplex PCR).Out of six samples, G genotypes of 3 samples were **G1 (50%)**, for remaining 3 samples G genotypes **G2** (50%). Out of six samples P genotypes of 3 samples were **P [4] (50%)**, P genotypes of 2 samples were P[8] **(33%)**, and for one sample resulted as partial genotyping (17%) due to there was no amplification for P genotyping.

G-P type combination most frequently found was G2P[4] (50%) followed by G1 P[8] (33%). Remaining 17% was untypable.

To know G-P Genotypes analysis pertaining to our geographical area is very usual to decide for selection of type of vaccine whether to select monovalent or pentavalent vaccine i.e. Rotarix RV1 or RotaTeq RV5 respectively. Since in our study G-P genotypes that is prevalent in Thanjavur tertiary care hospital were G2P[4] & G1 P[8] Pentavalent vaccine **RotaTeq RV5** is preferred over monovalent vaccine Rotarix RV1.

Similarly a study by Saravanan et al, a study in Chennai states that predominant strains was G2P[4].[98] In a previous study Babji et al, G1P[8], G2P[4] account for 63% of all genotypes and 7% remained untypable.[6] According to Arguelles et al G2P[4]was most frequently found.[78] All the above studies were similar to the present study in the prevalence of Genotypes.

In another study by Mathew et al, G1P[8] 49% was most common strain followed by G9P[8] and G2P[4].[91] According to Manohar Badur et al 56.25% of the Rotaviruses were of G1P[8] genotype followed by G12P[6].[99] In a study by Ion Guti´errez-Aguirre et al G1P[8] and G2P[4] was the predominant strains.[100] According to Iyoha and Abiodun he did only G genotyping in Nigeria states that G1 and G2 were common.[101] From all of the

above studies and the present study it is understood that genotypic prevalence varies from place to place and Rotavirus shows rapid strain variations. And also its very important to know the genotype information pertaining to particular region.

6.12 STRENGTH OF THE STUDY

- All aspects of etiological factors for acute gastroenteritis were analyzed such as bacterial investigations, parasitological investigations and viral investigations for the samples from 80 children less than 5 years.
- Uniform and strict criteria were followed for case selection.
- First study using Rota antigen ELISA test for Serological diagnosis of Rotavirus in Thanjavur medical college and hospital for 80 samples.
- Quantitative RNA identification was diagnosed by Rotavirus-A Real-time PCR assay for 15 samples.
- Genotyping of Rotavirus was done to know about the strain circulating in Thanjavur district. Since in our study G-P genotypes that is prevalent in Thanjavur tertiary care hospital were G2P[4] & G1 P[8] Pentavalent vaccine **RotaTeq RV5** is preferred over monovalent vaccine Rotarix RV1.

6.13 LIMITATIONS OF THE STUDY

- Speciation of *Escherichia coli* i.e. pathogenic or non pathogenic strains were not studied due to technical constraints.
- Study population is limited to a single centre.
- Study population is limited to lower socioeconomic group. Pattern of RVGE in middle and upper socioeconomic group were not studied.

6.14 SUGGESTIONS

- Early diagnosis of Rota antigen helps in proper management of RVGE.
- Also prevent nosocomial infections by control of infections from infected children.
- Also prevent misuse of antibiotics when Rotavirus detection done earlier.
- Prevention of RVGE by health education about safe water supply, proper sanitation, good hygiene, and vaccination.
- G-P Genotyping states both G2P[8] & G1P[4] were common in our geographical distribution. RotaTeq RV5 pentavalent vaccine is preferred over Rotarix RV1 which is for G1P[4].

CHAPTER 7: SUMMARY

The study was conducted at Thanjavur Medical College Hospital, Thanjavur over a period of July 2014 to June 2015. Study was about diarrhoea among children below 5 years with special reference to Rotavirus including detection and molecular characterization of Rotavirus in a tertiary care hospital, Thanjavur. The study conducted on 80 children diagnosed as having acute diarrhoeal disease and were admitted in pediatric diarrhoea ward in Thanjavur medical college and hospital. Stool samples were collected from all 80 children. Stool samples initially processed for macroscopic and microscopic examination for parasitic examination, followed by bacteriological examination through culture and biochemical tests. Then stool samples were stored in deep freezer in -20 degree celsius until tested for rotaviral antigen by ELISA method. Molecular detection Method (Rotavirus-A Real-time PCR assay) was done for 15 samples which are randomly selected 8 positive samples and 7 negative samples by ELISA for Rota antigen. All the six samples which were positive by Rotavirus-A Real-time PCR assay were further processed for genotyping by conventional multiplex PCR.

- Prevalence of Rotavirus diarrhoea in children less than five years was about 29%.
- High prevalence of RVGE of about 35% was seen in the age group of below 2 years. There was a significant difference between the age group of less than 2 years and age group of more than 2 years ($p<0.05$).
- Prevalence of RVGE in both sexes was equal, 26% in male children and 32% in female children. There was no significant difference between the sexes in this study ($p>0.05$).

- High Rota viral prevalence of about 36% i.e. 15 out of 23 positive cases is seen during winter months from October to March. There was significant difference of Rotavirus prevalence between winter months and other seasons ($p<0.001$).
- Next to diarrhoea the predominant symptom was vomiting which was seen in 87% of positive cases. Followed by fever was seen in 74% of positive cases. Decreased urine output was present in 13% of RV positive cases and abdominal pain was present in 8% of RV positive cases.
- Prevalence rate of dehydration in RVGE was about 38%. There was significant difference between dehydration positive cases and without dehydration positive cases ($p <0.05$).
- Prevalence of RVGE with regards to duration of hospitalization was about 100% in > 7 days of admission, about 30% prevalence in those who admitted for 3-7 days & 0% prevalence in those who admitted for less than 2 days. There was high significant difference of Rotavirus prevalence between long duration of hospitalization and short duration of hospitalization (p value <0.001).
- Prevalence of bacterial cause for diarrhoea below 5 years of age in this study was about 17%. In 8% cases rotaviral antigen was detected along with bacterial isolates. About 79% of the bacterial isolates were detected as E. coli.
- Prevalence of parasitic infections in this study was about 5%.
- Comparison between Rotavirus-A Real-time PCR & ELISA, 40% was positive by both ELISA & Rotavirus-A Real-time PCR. 46% were negative by both ELISA & Rotavirus-A Real-time PCR. 13% which were positive by ELISA and negative by RT PCR considered as borderline positives. There was significant difference

between these two tests in diagnosing Rotavirus infections in stool samples (p value < 0.05).

- G –P genotyping done for 6 samples which were positive by Rotavirus-A Real-time PCR. Among 6 samples, G-P genotype combination most frequently found was G2P[4] (50%) followed by G1 P[8] (33%). Remaining 17% was untypable. G-P genotypes that is prevalent in Thanjavur tertiary care hospital were G2P[4] & G1 P[8] hence Pentavalent vaccine **RotaTeq RV5** is preferred over monovalent vaccine Rotarix RV1.

CHAPTER 8: CONCLUSION

This study highlights the prevalence of Rota Viral Gastro Enteritis (RVGE) in under five year children which is 29%. Rotaviral gastroenteritis is common during winter months from October to January. RVGE was more common in less than 2 years of age. In RVGE dehydration is more common and severe when compared to non RVGE. Duration of hospitalization in RVGE is longer when compared to non RVGE. ELISA for detection of rotaviral antigen is the usual method for early diagnosis of RVGE and also eliminates indiscriminate use of antibiotics when it used early. RT PCR is gold standard method for diagnosing RVGE but it's very expensive to undertake as routine diagnostic procedure. The commonest strain of Rotavirus pertaining to Thanjavur tertiary care hospital during the study period was G2P[8] followed by G1P[4]. This study has made it clear that one-fourth of the diarrhoeal disorders among children aged under 5 years are due to Rotavirus, which calls for stringent preventive measures in terms of compulsory vaccination against Rotavirus. From the genotyping analysis in our geographical distribution pentavalent vaccine RotaTeq RV5 is preferred over monovalent vaccine Rotarix RV1. Also importance should also be given to the personal hygiene and hand washing practices of family members. Implementation of effective control measures such as safe drinking water, proper sanitation and vaccination is very much needs to control the morbidity caused by RVGE in under five children. Rotavirus vaccine is currently intensively targeted for inclusion in national immunization programme in India if it is included children in lower socioeconomic status will be benefited.

APPENDIX 1: DETAILS OF MEDIA AND REAGENTS PREPARATION

1. Iodine preparation for wet mount examination:

Potassium Iodide : 1 gram

Iodine : 1.5 gram

Distilled water : 100ml

2. Macconkey Agar:

5.5 gm dehydrated MacConkey agar is taken and 100 ml distill water is added. Then it is heated to dissolve completely. pH adjusted to 7.4. Then autoclaved at 121°C for 15 min.

3. Gram Stain:

Methyl violet:

Methyl violet – 1.5 gm

Distilled water – 100 ml

Grams iodine

Potassium iodide – 2 gm

Iodine - 2 gm

Water – 100 ml

Dilute Carbol Fuchscin:

Strong carbolfuchscin:

Basic fuchscin – 0.5 gm

Phenol crystal – 2.5 gm

Alcohol – 10 ml

Water - 90 ml

Take 1 ml of strong carbolfuchscin and add 19 ml of distilled water.

4. Indole Test:

Peptone water:

Peptone - 5gm

Sodium chloride – 2.5 gm

Water – 500 ml

Autoclave at 121°C for 15 minutes

5. Methyl Red Test / Voges Proskauer Test:

MR-VP broth:

1.7 gm of glucose phosphate broth is taken and 100 ml distilled water is added and it heated to dissolve completely. pH adjusted to 6.9. Then autoclaved at 121°C for 15 min. Then dispensed in separate tubes.

MR reagent:

Methyl red - 0.1 gm

95 % Ethyl alcohol – 300 ml

VP reagent 1:

α- naphthol – 5 gm

Absolute ethyl alcohol – 100 ml

VP reagent 2:

Potassium hydroxide – 40 gm

Distilled water – 100 ml

6. Citrate Utilization Test:

2.428 gm of dehydrated citrate powder is taken in a conical flask and 100 ml of distilled water is added. It was boiled till it dissolved completely. pH adjusted to 6.8. Then autoclaved at 121° C for 15 min.

7. Urease Test:

2.401 gm of dehydrated urea agar base is taken and 95 ml of distilled water is added. It was boiled to dissolve it completely. pH adjusted to 6.8. Then autoclaved at 121° C for 15 min and then cooled to 50° C. 5 ml of 40% of urease solution is added.

8. Triple Sugar Iron Agar:

6.452 gm of dehydrated medium is taken and mixed with 100 ml of distill water. Then it is heated to dissolve. pH is adjusted to 7.4.Then autoclaved at 121° C for 15 min. Then it is dispensed in test tubes in such a way to form a slant and butt.

APPENDIX 2: CONSENT FORM

தகவல் அறிந்து ஆய்வில் பங்கேற்பதற்கான ஒப்பந்தம்

நான் வயிற்றுப்போக்கு என்னும் நோயை பரப்பக்கூடிய ரோட்டா வைரஸ் கிருமிகளை கண்டுபிடிக்கும் ஆய்வில் ஈடுபட்டுள்ளேன்.

எனது பட்டபடிப்பின் ஒரு பகுதியாக எனது துறை பேராசிரியர்களின் வழிகாட்டுதலின் பேரில் தஞ்சை மருத்துவக்கல்லூரி மருத்துவமனையில் உள்ள நுண்ணுயிரியல் துறை ஆய்வகத்தில் இந்த ஆய்வினை மேற்கொள்கிறேன்.

இந்த ஆய்விற்காக உங்கள் குழந்தைகளிடமிருந்து மலம் மாதிரிகள் பெறப்படும். அந்த மாதிரிகள் யாருக்கும் பாதகம் இன்றி ஆய்விற்கு உட்படுத்தப்படும். இந்த ஆய்வினால் உங்களுக்கு எந்த தீங்கும் ஏற்படாது எனவும் நீங்கள் இந்த ஆய்விற்கு எந்த நேரத்திலும் பங்கு கொள்ளாமல் விலகிக் கொள்ளலாம் என்பதையும் தெரிவித்துக்கொள்கிறேன்.

ஆய்வாளரின் கையொப்பம்

தேதி :

ஆய்வுக்குட்பட்டகுழந்தையின்
பெற்றோர் கையொப்பம்

தேதி:

முகவரி:

APPENDIX 3: PROFORMA

PROFORMA

NAME:
AGE:
SEX:
FATHER'S NAME:
ADDRESS:

SERIAL NO:
LAB NO:
OP/IP NO:
ADMISSION DATE:
DISCHARGE DATE

FATHER'S OCCUPATION:

DATE OF STOOL COLLECTION:

FAMILY INCOME:

CHIEFS COMPLAINTS:

1. DIARRHOEA
2. FEVER
3. VOMITING
4. ABDOMINAL PAIN
5. OTHERS

PAST HISTORY:

1. PREVIOUS EPISODES
2. BREAST FEEDING
3. WEANING H/O
4. VACCINATION H/O

FAMILY HISTORY:

GENERAL EXAMINATION:

CLINICAL DIAGNOSIS:

APPENDIX 4: MASTER SHEET

S No	Month	Age in months	Sex	Dehydration	Diarrhoea	Fever	Vomiting	↓ Urine Output	Parasite /ova cyst	Bacterial Isolates	Other symptoms	Duration of stay	ELISA rota Ag Result	RT PCR	Genotyping
1	June	2	mch	Severe	Y	Y	Y					3	-		
2	June	42	fch	Some	Y	Y	Y			E. coli		4	-		
3	June	4	mch	No	Y	Y	-					4	-		
4	June	9	mch	Some	Y	Y	Y					3	-	-	
5	June	36	fch	No	Y	Y	-		Y			4	-		
6	June	28	mch	Some	Y	Y	Y					5	-		
7	July	36	fch	Some	Y	Y	Y			E. coli		2	-		
8	July	3	fch	Some	Y	Y	Y				Abdominal pain	7	+	+	G1P[8]
9	July	5	fch	Some	Y	Y	Y					8	+		
10	July	30	mch	No	Y							2	-	-	
11	August	58	mch	Severe	Y	Y	Y					5	+		
12	August	12	fch	Some	Y	Y				E. coli	Septicaemia	5	-		
13	August	26	fch	No	Y							3	-		
14	August	36	fch	No	Y	Y			Y			3	-		
15	September	9	mch	Some	Y	Y	Y					6	+		
16	September	9	fch	Some	Y	Y	Y					5	-		
17	September	36	mch	No	Y	Y						1	-		
18	September	36	mch	Some	Y	Y	Y			E. coli		4	-	-	
19	September	28	mch	No	Y							1	-		
20	September	12	mch	Some	Y	Y	Y					2	-		
21	September	5	fch	No	Y	Y	Y					3	-		
22	October	36	fch	Some	Y	Y	Y					4	+	+	G2P[4]
23	October	1	mch	Some	Y	Y	Y					5	-		
24	October	27	mch	No	Y		Y					2	-		
25	October	18	mch	No	Y	Y						2	-		
26	October	3	fch	Severe	Y	Y	Y					5	+		
27	November	2	mch	No	Y		Y					3	-		
28	November	12	mch	Some	Y	Y	Y			E. coli		5	+		
29	November	9	fch	No	Y							2	-		
30	November	12	mch	Some	Y		Y					4	-	-	
31	November	24	mch	No	Y					E. coli		3	+		
32	November	26	fch	No	Y		Y					2	-		
33	November	12	fch	No	Y	Y					Abdominal pain	3	-		

S No	Month	Age in months	Sex	Dehydration	Diarrhoea	Fever	Vomiting	↓ Urine Output	Parasite /ova cyst	Bacterial Isolates	Other symptoms	Duration of stay	ELISA rota Ag Result	RT PCR	Genotyping
34	November	18	fch	No	Y		Y					5	+		
35	November	12	mch	No	Y		Y					2	-		
36	November	30	fch	Some	Y		Y			*Klebsiella*		2	-		
37	November	12	mch	Severe	Y	Y	Y	Y			↓Urine output	7	+	+	G2P[4]
38	November	27	mch	Some	Y	Y	Y					1	-		
39	December	12	fch	No	Y							2	-		
40	December	24	fch	Some	Y	Y	Y					3	+		
41	December	11	fch	Severe	Y	Y	Y			*E. coli*	Abdominal pain	3	-		
42	December	5	fch	Some	Y		Y					2	-		
43	December	9	fch	No	Y							5	+		
44	December	18	fch	Some	Y		Y					3	-		
45	December	11	mch	No	Y							2	-		
46	December	58	mch	Some	Y		Y			*Shigella sp*		5	-	-	
47	December	18	mch	Some	Y	Y						3	-		
48	December	18	mch	Some	Y	Y	Y					7	+		
49	December	12	fch	No	Y	Y				*E. coli*		2	-		
50	December	40	mch	No	Y				Y			2	-		
51	December	5	mch	Severe	Y	Y	Y				Abdominal pain	6	+		
52	January	12	fch	Some	Y	Y	Y					5	+		
53	January	24	mch	Some	Y	Y	Y					7	+		
54	January	24	mch	Some	Y	Y	Y	Y			↓Urine output	6	+	+	G1P[8]
55	January	7	mch	No	Y	Y					URI	3	-		
56	February	10	mch	Severe	Y	Y	Y				Abdominal pain	3	-		
57	February	10	fch	Severe	Y	Y	Y	Y			↓Urine output	8	+	+	G1
58	February	10	mch	No	Y	Y						2	-		
59	February	8	mch	No	Y		Y					2	-		
60	February	18	mch	No	Y						LRI	3	-		
61	March	7	mch	Severe	Y	Y	Y			*E. coli*	Shock	5	-	-	
62	March	16	mch	No	Y							4	+		
63	March	15	fch	Some	Y	Y	Y					2	-		
64	April	11	mch	Severe	Y	Y	Y			*E. coli*	Septicaemia	6	-		
65	April	18	mch	No	Y							2	-		
66	May	59	mch	No	Y		Y		Y			2	-		
67	May	1	mch	Some	Y	Y	Y					2	-		
68	May	38	fch	No	Y	Y						2	-		

S No	Month	Age in months	Sex	Dehydration	Diarrhoea	Fever	Vomiting	↓ Urine Output	Parasite /ova cyst	Bacterial Isolates	Other symptoms	Duration of stay	ELISA rota Ag Result	RT PCR	Genotyping
69	May	5	mch	No	Y	Y	Y					3	+	-	
70	May	6	mch	No	Y	Y	Y					3	-		
71	May	12	mch	no	Y	Y	Y					8	+	+	G2P[4]
72	May	18	mch	Some	Y	Y						4	-	-	
73	May	30	fch	No	Y		Y			*Klebsiella*		2	-		
74	May	8	mch	Some	Y		Y					3	-		
75	May	11	fch	No	Y	Y	Y					2	-		
76	May	54	fch	Some	Y		Y					5	+	-	
77	June	10	mch	No	Y							3	-		
78	June	12	fch	Some	Y		Y			*E. coli*		2	-		
79	June	24	fch	No	Y		Y					3	+		
80	June	54	fch	No	Y		Y					4	-		

Mch - Male Child
Fch - Female Chile
Y - Yes
N - No
\+ - Positive
\- - Negative

BIBLIOGRAPHY

[1] MORRIS, K. et al. (2012) Rotavirus mortality in India: estimates based on a nationally representative survey of diarrhoeal deaths. *Bulletin of the World Health Organization*. 90.p. 720-727.

[2] TANEJA, K.& AKASH MALIK.(2012) Burden of Rotavirus in India - Is Rotavirus Vaccine an Answer to It? *Indian Journal of Public Health*. 56(1/Jan).p.17-21.

[3] PARASHAR, D.et al. (2009) Global Mortality Associated with Rotavirus Disease among Children in 2004. *The Journal of Infectious Diseases*. 200 (supp 1).p.S9–15.

[4] JOHN et al. (2014) Rotavirus gastroenteritis in India, 2011–2013: Revised estimates of disease burden and potential impact of vaccines. *Elsevier Ltd, Vaccine* [online]. 32S.p.A5–A9. Available from http://www.sciencedirect.com/science/journal/0264410X. [accessed on 10/5/2015]

[5] KURUQOL, Z .et al. (2003) Rotavirus gastroenteritis among children under five of age in Izmir, Turkey. *Turkey J pediatrics*. 45(4 / Oct-Dec).p.290-4.

[6] BABJI, S. et al. (2013) Detection and characterization of rotaviruses from children less than 5 years hospitalized with acute gastroenteritis in Nagercoil. *Indian Journal of Medical Microbiology*. 31(1/ January-March).p.69-71.

[7] CHAVAN, C. et al. (2013) Prevalence of rotavirus diarrhoea among children hospitalized in a tertiary care hospital in Western India. *Int J Pharm Biomed Sci* .4(1).p.4-7.

[8] SAIA. et al. (2010) Hospitalization for rotavirus gastroenteritis in the pediatric population in the Veneto Region, Italy. *BMC Public Health*, [online].10.p.636. Available from http://www.biomedcentral.com/1471-2458/10/636. [accessed on 19/06/2015]

[9] SAI .et al.(2013)Epidemiology and clinical features of rotavirus and norovirus infection among children in Ji'nan, China. *Virology Journal*, [online].10.p.302.Available from http://www.virologyj.com/content/10/1/302. [accessed on 19/06/2015]

[10] ADAMS, W. R., and KRAFT, L.M. (1967) Electron-Microscopic Study of the Intestinal Epithelium of Mice Infected with the Agent of Epizootic Diarrhoea of Infant Mice (EDIM Virus). *Am J Path*.51(1/Jul).p.39-60.

[11] BISHOP, R. (2009) Discovery of rotavirus: Implications for child health. *Journal of gastroenterology & hepatology*.24 (Suppl 3 /Oct).p.S81-5.

[12] BISHOP, RF., DAVIDSON, GP., HOLMES,IH., & RUCK, BJ. (1973) Virus particles in epithelial cells of duodenal mucosa from children with acute non-bacterial gastroenteritis. *Lancet*.2 (7841/Dec).p.1281–1283.

[13] DAVIDSON, G. P. et al. (1975) Importance of new virus in acute sporadic enteritis in children. *Lancet*. 1.p.242.

[14] FLEWETT,T.H.et al. (1974) Relation between viruses from acute gastroenteritis of children and newborn calves.*Lancet*.2.p.61-63.

[15] BISHOP, R.F.,DAVIDSON, G.P., HOLMES, I.H.,RUCK,B.J.(1974) Detection of a new virus by electron microscopy of faecal extracts from children with acute gastroenteritis. *Lancet*. 1(7849/Feb).p.149–151.

[16] HOLMES, I. H., et al.(1974) Orbivirus & gastroenteritis.*Lancet*.2.p.658-9.

[17] UNITED STATES.CENTERS FOR DISEASE CONTROL AND PREVENTION. Epidemiology & Prevention of Vaccine-Preventable Diseases.(2015).*Rotavirus CDC guidelines.*13th Edition. Atlanta: CDC. (April/311-323).

[18] MICHAEL CAMILLERI, & MURRAY, A. (2012) *Harrison s principles of Internal Medicine.* Diarrhoea and constipation.18th edition. New York: Mcgraw-Hill.p.308-316.

[19] SHILPI GUPTA, et al.(2015) Aetiology of childhood viral gastroenteritis in Lucknow, North India. *Indian J Med Res.*141(Apr).p.469-472.

[20] ZULFIQAR AHMED BHUTTA. (2008) *Nelson textbook of paediatrics*. Acute gastroenteritis in children.18th edition.Philadelphia:saunders.p.1605-1617.

[21] SHETTY, et al. (2014) Rotavirus and Adenovirus diarrhoea in children. *Muller Journal of Medical Science and Research*. 5 (2/Jul – Dec).p.143-148.

[22] KAPIKIAN, A. Z., & CHANOCK, R. M.(1996) *B. N. Fields In Virology*. Rotaviruses. Philadelphia: Lippincott-Raven.p.1657-1708.

[23] ESTES, K . & GREENBERG,B.(2013).*Field ’s virology*. Rotaviruses vol 2 .6th edition. Philadelphia: Lippincott Williams.

[24] PRASAD,B.V.V.et al.(1988)Three-dimensional structure of rotavirus. *Journal of Molecular Biology*.199(2/Jan).p.269-275.

[25] PRASAD,et al.(1996)Visualization of ordered genomic RNA and localization of transcriptional complexes in rotavirus. *Nature*.382 **(**Aug**)**.p.471-473.

[26] CRAWFORD,et al.(1999)Characterization of Virus-Like Particles Produced by the Expression of Rotavirus Capsid Proteins in Insect Cells. *Journal of virology*. 68 (9/sep).p. 5945-5952.

[27] ULRICH DESSELBERGER,JIM GRAY,MARY K.ESTES.(2005) *Topley & Wilson’s Microbiology & Microbial infections.*Rotaviruses.10th edition. London: Hodder Arnold.

[28] ESTES,M.K.,& COHEN,J.(1989) Rotavirus gene structure and function. *Microbiol Rev.*53(4/Dec).p.410–449.

[29] JAWETZ, MELNICK, & ADELBERG.(2013).*Medical Microbiology*. Reoviruses, Rotaviruses, and Caliciviruses.26th edition.New York:McGraw-Hill.

[30] ITURRIZA GOMARA, et al.(2002)Molecular Characterization of VP6 Genes of Human Rotavirus Isolates: Correlation of Genogroups with Subgroups and Evidence of Independent Segregation. *Journal of virology*.76(13/ July).p.6596–6601.

[31] THEIL,et al.(1985) Porcine Rotavirus-Like Virus (Group B Rotavirus): Characterization and Pathogenicity for Gnotobiotic Pigs. *J. Clin. Microbiol*.21 (3/Mar).p.340-345.

[32] GREENBERG, et al.(1983) Serological Analysis of the Subgroup Protein of Rotavirus, Using Monoclonal Antibodies. *Infect. Immun*.39 (1/Jan).p.91-99.

[33] RODRIGUEZ CASTILLO,et al.(2000)VP4 and VP7 Genotyping by Reverse Transcription-PCR of Human Rotavirus in Mexican Children with Acute Diarrhoea. *J. Clin.microbiol*.38(10/Oct).p.3876–3878.

[34] SANKAR SURENDRAN.(2008) Rotavirus infection: molecular changes and pathophysiology .*EXCLI Journal*.7(Sep).p.154-162.

[35] LUDERT,E. et al.(1996)Cleavage of rotavirus VP4 in vivo. *Journal of General Virology*.77 (Nov).p.391- 395.

[36] CRAWFORD,et al.(2001)Trypsin Cleavage Stabilizes the Rotavirus VP4 Spike. *Journal of virology.* 75(13/July).p.6052–6061.

[37] CUADRAS,et al.(2002) Gene Expression Pattern in Caco-2 Cells following Rotavirus Infection. *Journal of virology*. 76(9/May).p.4467–4482.

[38] RUIZ, M.C. et al.(1997) Cell lines susceptible to infection are permeabilized by cleaved and solubilized outer layer proteins of rotavirus. *Journal of General Virology*.78(11/ Jun).p.2883–2893.

[39] YIZHI TAO,et al. (2002).RNA Synthesis in a Cage Structural Studies of Reovirus Polymerase lambda 3.*Cell.*111(Nov).p.733-745.

[40] VENDE,et al.(2000) Efficient Translation of Rotavirus mRNA Requires Simultaneous Interaction of NSP3 with the Eukaryotic Translation Initiation Factor eIF4G and the mRNA 39 End .*Journal of virology*.74(15/Aug).p.7064–7071.

[41] ELSA FABBRETTI,et al.(1999)Two non-structural rotavirus proteins, NSP2 and NSP5, form viroplasm-like structures in vivo.*Journal of General Virology*.80(Oct).p.333–339.

[42] JOURDAN,et al.(1997) Rotavirus Is Released from the Apical Surface of Cultured Human Intestinal Cells through Nonconventional Vesicular Transport That Bypasses the Golgi Apparatus. *Journal of virology*.71(11/Nov).p.8268–8278.

[43] SAPIN, et al.(2002) Rafts Promote Assembly and Atypical Targeting of a Nonenveloped Virus, Rotavirus, in Caco-2 Cells. *Journal of virology*.76(9/May).p.4591-4602.

[44] OBERT.et al.(2000) Rotavirus-Induced Structural and Functional Alterations in Tight Junctions of Polarized Intestinal Caco-2 Cell Monolayers. *Journal of virology*.74(10/May).p.4645–4651.

[45] STINTZING,G.et al.(1986) Intestinal permeability in small children during and after rotavirus diarrhoea assessed with different-size polyethyleneglycols (PEG 400 and PEG 1000*). Acta Paediatr Scand*.75(6/Nov).p.1005-9.

[46] SALIM, A. F,et al.(1995)Sequential changes in small intestinal structure and function during rotavirus infection in neonatal rats.*Gut.* 36.p.231-238.

[47] ASHWIN BORADE.(2010) characteristics of rotavirus gastroenteritis in hospitalized children in pune.*Indian Journal of Medical Sciences.*64(5 /May).p.210-218.

[48] BERNARD BURKE & ULRICH DESSELBERGER.(1996)Rotavirus Pathogenicity. *Virology*.218(Feb).p.299–305.

[49] BOSHUIZEN ,J.A.(2005) *Pathogenesis of Rotavirus Infection*.phD thesis. Erasmus University ,Rotterdam.

[50] BALL, J.M.et al .(1996) Age-Dependent Diarrhoea Induced by a Rotaviral Nonstructural Glycoprotein. *Science magazine*.272(5258/Apr).p.101-104.

[51] PAGE et al.(2010) Emergence and Characterization of Serotype G9 Rotavirus Strains from Africa.*The Journal of Infectious Diseases*.202(S1).p.S55–S63.

[52] ASMAH.et al.(2001)Rotavirus G and P Genotypes in Rural Ghana. *J. Clin.microbio.* 39(5/May).p.1981–1984.

[53] MOHAMMAD KARGAR, MARYAM ZARE, AKRAM NAJAFI.(2012)Molecular Epidemiology of Rotavirus Strains Circulating among Children with Gastroenteritis in Iran. *Iran J Pediatr*.22(1/Mar).p.63-69.

[54] BAHL et al.(2005) Incidence of Severe Rotavirus Diarrhoea in New Delhi, India, and G and P Types of the Infecting Rotavirus Strains. *The Journal of InfectiousDiseases*. 192.p.S114–9.

[55] KANG, et al.(2005) Epidemiological Profile of Rotaviral Infection in India: Challenges for the 21st Century. *The Journal of Infectious Diseases.*192(suppl 1).p.S120–S126.

[56] DAS et al.(2003) Emergence of Novel Human Group A Rotavirus G12 Strains in India . *J. Clin.Microbiol*.41(6/Jun).p.2760–2762.

[57] ALRIFAI,S.B.,et al.(2009) Prevalence and etiology of nosocomial diarrhoea in children < 5 years in Tikrit teaching hospital. *Eastern Mediterranean Health Journal*.15(5/Jul).p.1111-1118.

[58] EZEONWU, et al.(2013) Clinical Features of Acute Gastroenteritis in Children at University of Nigeria Teaching Hospital, Ituku‑Ozalla, Enugu. *Annals of Medical and Health Sciences Research*. 3(3 /Jul-Sep).p.361-364.

[59] KAPIKAN ,et al.(1983) Oral Administration of Human Rotavirus to Volunteers: Induction of Illness and Correlates of Resistance. *J Infect Dis*.147(1).p.95-106 .

[60] COOK, S.M. et al.(1990) Global seasonality of rotavirus infection. *Bulletin of the World Health Organization.*66(2).p.171-177 .

[61] GRAY, et al.(2008)Rotavirus. *Journal of pediatric Gastroenterology and Nutrition.* 46(suppl 2/ May).p.s24-s31.

[62] LOGAN et al.(2006) Real-Time Reverse Transcription-PCR for Detection of Rotavirus and Adenovirus as Causative Agents of Acute Viral Gastroenteritis in Children. *J. CLIN.MICROBIOL*.44(9/ Sep).p.3189–3195.

[63] HEMMING, et al. (2014)Rotavirus Antigenemia in Children is Associated With More Severe Clinical Manifestations of Acute Gastroenteritis. *The Pediatric Infectious Disease Journal*.33(4/Apr).p.366-371.

[64] STEVE PEDLEY, et al.(1984)The Genomes of Rotaviruses Isolated from Chronically Infected Immunodeficient Children. *J. gen. Virol*.65(Mar).p.1141- 1150.

[65] VELAZQUEZ ,et al. (1996)Rotavirus infection in infants as protection against subsequent infections. *The New England Journal of Medicine*.335(14/Oct).p.1022-1028.

[66] SWITZERLAND.WORLD HEALTH ORGANIZATION. .(2009) *Manual of rotavirus detection and characterization methods*.Geneva:WHO publications.(p.146 Report No.: WHO/IVB/08.17.)

[67] KNISLEY et al.(1986) Detection of Rotavirus in Stool Specimens with Monoclonal and Polyclonal Antibody-Based Assay Systems.*J. Clin. Microbiol*.23(5/May).p.897-900.

[68] JAMES et al.(1998) Enzyme-Linked Immunosorbent Assay Based on Recombinant Human Group C Rotavirus Inner Capsid Protein (VP6) To Detect Human Group C Rotaviruses in Fecal Samples.*Journal of clinical microbiology.*36(11/ Nov).p.3178–3181 .

[69] RAY et al.(2006) Quantitative Evaluation of Rotaviral Antigenemia in Children with Acute Rotaviral Diarrhoea.*J Infect Dis.*194(Sep).p.588-594**.**

[70] KELKAR et al.(2004) Rapid ELISA for the diagnosis of rotavirus.*Indian J Med Res*. 119(Feb).p.60-65.

[71] PEREIRA, RABONI, NOGUEIRA et al. (2011)Rotavirus infection in a tertiary hospital: laboratory diagnosis and impact of immunization on pediatric hospitalization. *Braz J Infect Dis.*15(3/Feb).p.215-219.

[72] MATHUR, M.S & BHAVE,G.G. (1993) comparative evaluation of Coagglutination and Latex agglutination(LA) for detection of rota virus.*JPGM*.39(3).p.130-133.

[73] RABONI et al.(2002) Comparison of Latex Agglutination With Enzyme Immunoassay for Detection of Rotavirus in Fecal Specimens.*Am J Clin Pathol*.117.p.392-394.

[74] DZIKWI, A. et al.(2008) Electropherotypes and subgroups of group A rotaviruses circulating among diarrhoeic children in Kano, Nigeria.*Ann Afr Med* .7(4).p.163-167.

[75] MUSTAFA ALTINDIS, et al.(2004)Rotavirus Infection in Children with Acute Diarrhoea as Detected by Latex Agglutination, ELISA and Polyacrylamide Gel Electrophoresis. *Indian pediatrics*.41(Jun).p.590-594.

[76] DOLAN ,et al.(1985) Epidemiology of Rotavirus Electropherotypes Determined by a Simplified Diagnostic Technique with RNA Analysis. *J. Clin. Microbiol*.21(5/May).p.753-758.

[77] VENKATESH V.N. et al.(2012)Rotaviral Diarrhoea in Children A Comparison of PAGE with ELISA. *Journal of Clinical and Diagnostic Research*.6(2/Apr).p.188-191.

[78] ARGU ELLES, et al. (2000)VP7 and VP4 Genotyping of Human Group A Rotavirus in Buenos Aires, Argentina. *J. Clin.microbiol*.38(1/Jan).p.252–259.

[79] ANDI YASMON, et al. (2010)Detection of human group a and c rotaviruses in pediatric patients with acute gastroenteritis by real time rt-PCR assay:a preliminary study. *J Makara, kesehatan*.14(2/Dec).p.65-69.

[80] DRISKELL, et al. (2010)Rapid and Sensitive Detection of Rotavirus Molecular Signatures Using Surface Enhanced Raman Spectroscopy.*PLoS ONE* [online].5(4).e10222.Available from http://www.plosone.org**.** [Accessed 22/08/2015].

[81] VESIKARRI,T.(1990)Rotavirus Disease in Finnish Children: Use of Numerical Scores for Clinical Severity of Diarrhoeal Episodes. *J Infect Dis*.22(3).p.259-267.

[82] TELMESANI, M.A. (2010)Oral rehydration salts, zinc supplement and rota virus vaccine in the management of childhood acute diarrhoea. *Journal of Family and Community Medicine*.17(2/Aug).p.79-82.

[83] GENEVA.WHO/UNICEF JOINT STATEMENT. (2004) *Clinical management of acute diarrhoea*.Switzerland:WHO publications.(Report No.:WHO/FCH/CAH/04.7)

[84] ATLANTA. CENTERS FOR DISEASE CONTROL AND PREVENTION.(2003) .*Managing Acute Gastroenteritis Among Children*. MMWR publications.Nov. 52 (RR-16).p.1- 20.

[85] MELINDA, K.& CHRISTA, L. (2010 The effect of rotavirus vaccine on diarrhoea mortality. *International Journal of Epidemiology*.39.p.i56–i62.

[86] VIKAS BAJPAI, et al.(2012) Agenda Setting in Vaccine Policy and Social Relevance of the Emerging Vaccine Technologies From Public Health Perspective – PART 2. *International Journal of Medicine and Public health.*2(1).p.16-25.

[87] India Rotavirus Vaccine Group. (2014) Efficacy of a monovalent human-bovine (116E) rotavirus vaccine in Indian infants: a randomised, double-blind, placebo-controlled trial. *The lancet.*383(9935).p.2136-2143.

[88] KATOCH,V.M. (2014) Department of Health Research, Indian Council of Medical Research, New Delhi, India data for action : Indian Rotavirus Strain Surveillance Network.*vaccine* .32.p.SA1-A2.

[89] KANG et al. (2009) Multicenter, Hospital-Based Surveillance of Rotavirus Disease and Strains among Indian Children Aged <5 Years. *The journal of infectious Diseases*.200(1).p.S147-S153.

[90] MANOHAR, et al. (2015) Prevalence of Rotavirus diarrhoea among under-5 hospitalized children in a Government Tertiary Hospital, Tirupati. *Journal of Dr. NTR University of Health Sciences.4(2).p. 112-116*

[91] MATHEW, et al. (2013) Prevalence of rotavirus diarrhoea among hospitalizes children less than 5 years in Kerala, south India. *J Indian pediatrics*. sep.p.1-12.

[92] SHARIFF,M. et al. (2003) A study of diarrhoea among children in Eastern Nepal with special reference to rotavirus. *Indian journal of medical microbiology*.21(2/Apr).p.87-90.

[93] KAFI,SK. et al.(2013) Rotavirus infection among children under 5 years presenting with diarrhoea to Ahmed Gasim pediatric hospital,Khartoum. *Neelain Medical Journal.*3(9/Mar).p.ISSN1858-627.

[94] BLACK,R.E. et al. Two year study of bacterial,viral,and parasitic agents associated with diarrhoea in rural bangalaesh. *Journal Of Infectious Diseases.*142(2).p.660-664.

[95] BIJAY RANJAN MIRDHA .(2002)Hymenolepis nana: A Common Cause of Pediatric Diarrhoea in Urban Slum Dwellers in India. *J Trop Pediatr* .48 (6).p.331-334.

[96] XIAOLI, L.PANG,et al.(2004) Increased detection of rotavirus using a real time reverse transcription-polymerase chain reaction (RT-PCR) assay in stool specimens from children with diarrhoea. *Journal medical virology.*72(3/Mar).p.496-501.

[97] BUESA.J. et al. Evaluation of reverse transcription and polymerase chain reaction (RT/PCR) for the detection of rota viruses. *Research in virology*.147(6).p.353-361.

[98] SARAVANAN et al.(2004) Rotavirus infection among infants and young children in Chennai, south India. *Indian Journal of Medical Microbiology.* 22 (4/Oct).p.212-221.

[99] MANOHAR ,et al.(2015) Prevalence of rotavirus diarrhoea among children in Tirupati. *Journal of Dr. NTR University of Health Sciences. 4(2).p.112-116.*

[100] GUTIERREZ-AGUIRRE et al.(2008) Sensitive Detection of Multiple Rotavirus Genotypes with a Single Reverse Transcription–Real-Time Quantitative PCR Assay. *Journal of clinical microbiology*.46(8/Aug).p.2547–2554.

[101] O IYOHA, PO ABIODUN. (2015) Human rotavirus genotypes causing acute watery diarrhoea among under-five children in Benin City, Nigeria. *Nigerian Journal of Clinical Practice.*18(1/ Jan).p.48-51.